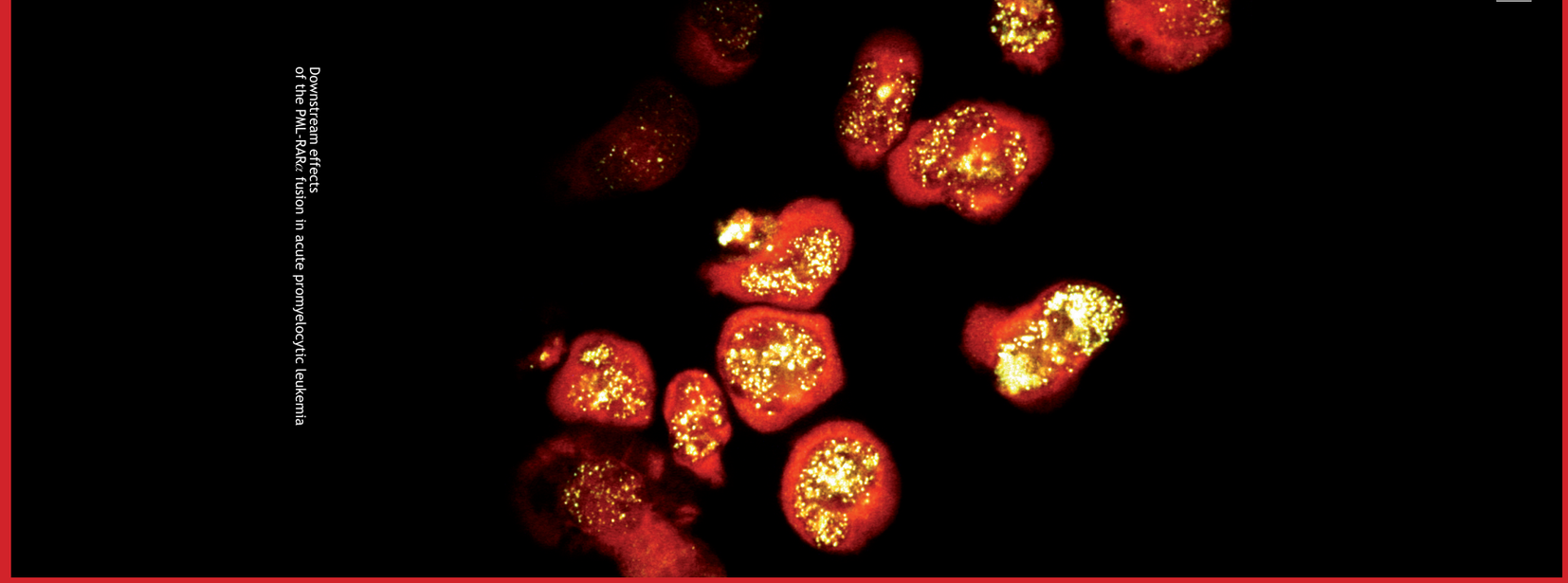

Downstream effects
of the PML-RARα fusion in acute promyelocytic leukemia

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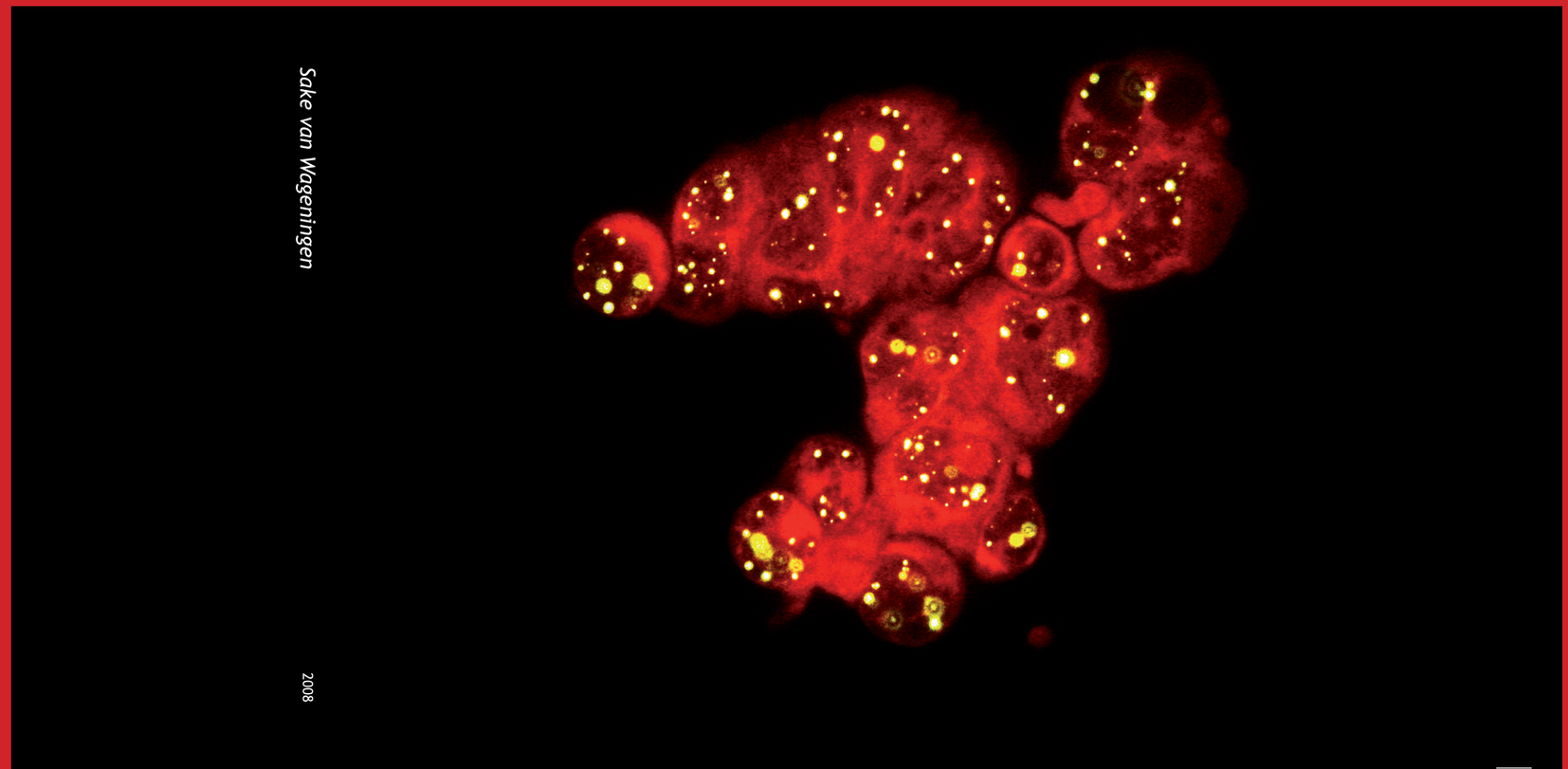
Sake van Wageningen



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The studies described in this thesis were performed between 2001 and 2006 at the Central Hematology Laboratory at the Radboud University Nijmegen Medical Centre, Nijmegen Centre for Molecular Life Sciences, Nijmegen, The Netherlands.

Downstream effects of the PML-RARα fusion

in

acute promyelocytic leukemia

Een wetenschappelijke proeve op het gebied van de

medische wetenschappen

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Voor mijn ouders

General introduction

Hematopoiesis

Every day the human body generates high numbers of new blood cells (Ogawa, 1993). Blood cells arise from immature cells (blasts) that reside in the bone marrow, a process called hematopoiesis (Figure 1). In the bone marrow, the immature cells proliferate, both to supply the body with cells that can mature into functional blood cells (differentiation) and to keep a constant pool of immature cells (self-renewal) (Fisher, 2002; Zon, 1995; Ogawa, 1993). The pluripotent stem cell population has been defined by its ability to reconstitute hematopoiesis in serial bone marrow transplantations in mice (Bertoncello et al., 1988; Lerner and Harrison, 1990; Ploemacher and Brons, 1989). *In vitro* colony forming unit (CFU) assays have identified various hematopoietic precursor cells that are able to generate a restricted number of blood cell types (McCulloch et al., 1978; McNiece et al., 1988; Moore et al., 1979; Nakahata and Ogawa, 1982). Current studies mainly use the expression of cell surface markers to purify specific cell populations. The stem cell can be distinguished by the lack of lineage

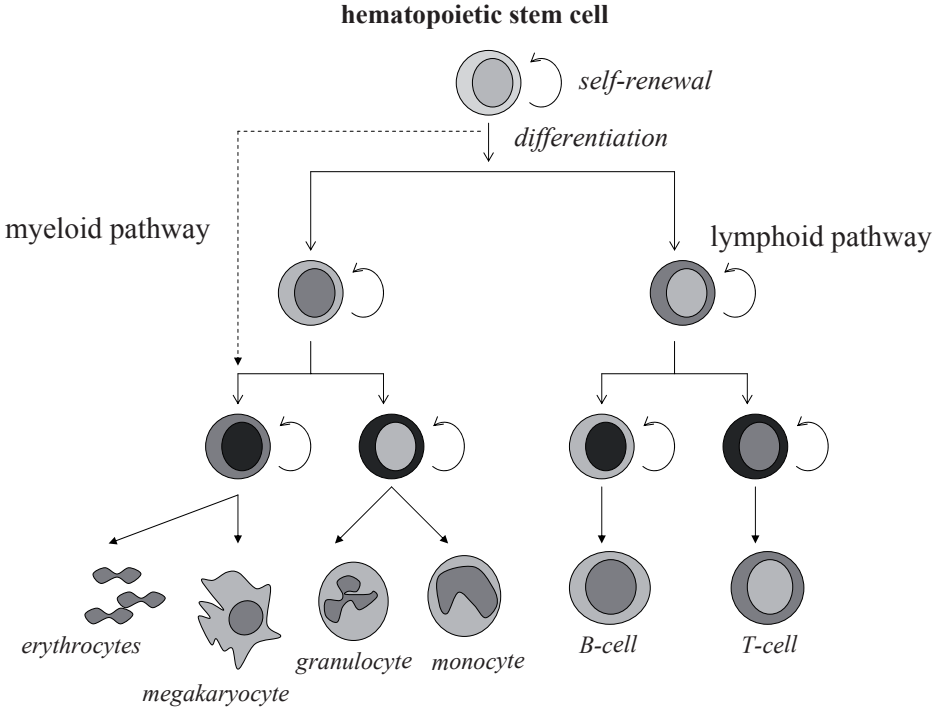

Figure 1. Hematopoiesis. Hematopoietic stem cells, residing in the bone marrow, supply cells that mature along different lineages. Cells mature along the myeloid lineage to become erythrocytes (red blood cells), megakaryocytes (and platelets), granulocytes or monocytes. Cells mature along the lymphoid lineage to become T-cells or B-cells. Alternatively, stem cells may differentiate into megakaryocyte/erythrocyte progenitors directly (dotted line).

markers and the presence of CD133 (Human cells, $Lin^{-}CD133^{+}$) or c-kit and Sca-1 (murine cells, $Lin^{-}Ska^{+}Kit^{+}$) (Wognum et al., 2003). Investigations into the lineage potential have suggested the existence of long- and short-term hematopoietic stem cells, common lymphoid progenitors (Kondo et al., 1997) and common myeloid progenitors (Akashi et al., 2000). The common myeloid progenitors differentiate into common granulocyte/monocyte progenitors and megakaryocyte/erythrocyte progenitors. Alternatively, recent studies suggest that the megakaryocyte/erythrocyte progenitor may originate from the short-term repopulating stem cell directly (figure 1, dotted line) (Adolfsson et al., 2005). The progenitors may mature into several types of specialised blood cells, to execute their function in immunity (defence against infections), coagulation (blood clotting) or transport of molecules like oxygen.

During life, cells may acquire genetic damage. Genetically damaged, immature blasts may be blocked in their differentiation and these cells may fill up the bone marrow and displace normal hematopoietic cells. This thesis will focus on a subtype of acute myeloid leukemia (AML). AML is defined by a more than 20% increase of blasts in the bone marrow.

AML

Several types of leukemia can be distinguished, depending on what type of blood cell is blocked in its differentiation. Cells can originate from the lymphoid lineage, resulting in chronic or acute lymphoid leukemia (respectively CLL or ALL), or from the myeloid lineage, resulting in chronic or acute myeloid leukemia (respectively CML or AML) (Redaelli et al., 2003). These types of leukemia are again subdivided, depending on the maturation stage of the leukemic cells. In the French-American-British (FAB) classification, AML is subdivided in 8 types (AML-M0 to AML-M7 (Lowenberg et al., 1999). Recently, the World Health Organization (WHO) has developed a new classification of hematopoietic neoplasms (Vardiman et al., 2002). This classification includes many of the criteria of the FAB-classifications, which rely on morphologic and cytochemical features, but also includes immunophenotypic features and recurring genetic abnormalities.

Genetic abnormalities in AML

AML cells often contain chromosomal translocations (Figure 2). Translocations may result in the fusion of genes that are located at the chromosomal breakpoints. Recurrent translocations that are found in AML include the t(8;21) translocation, resulting in the AML-ETO fusion, the t(15;17) translocation, resulting in the PML-RARα fusion, the t(16;16) translocation or inversion 16, resulting in the CBFβ-MYH11 fusion (Gilliland et al., 2004; Mrozek et al., 2004; Vardiman et al., 2002). There is a strong correlation between certain genetic abnormalities and the morphology of the leukemic blasts. Genetic abnormalities may be used to predict the response to certain therapies (Vardiman et al., 2002).

The genes affected by translocations are often involved in the development and homeostasis of the normal blood cells and the abnormal proteins deregulate proliferation,

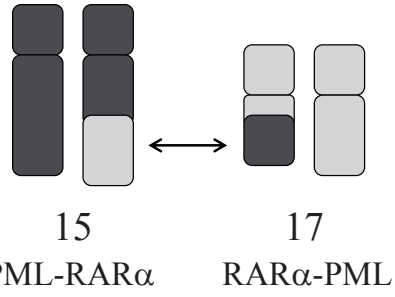

Figure 2. Chromosomal translocations. Chromosomes may exchange parts of their arms. Breakpoints of chromosomes found in APL are almost always found in the PML gene on chromosome 15 and in the RARα gene on chromosome 17. This translocation results in two fusion genes: PML-RARα and RARα-PML.

differentiation or programmed cell death (apoptosis) (Taki and Taniwaki, 2006; Mrozek et al., 2004; Scandura, 2005). Many of these genes encode for transcription factors. Study of these transcription factors have shown that they are essential in differentiation and lineage commitment during hematopoiesis (Tenen, 2003; Metcalf, 1993; Shivdasani and Orkin, 1996; Zhu and Emerson, 2002).

In addition, transcription factors involved in hematopoiesis and leukemia have been identified, which are not part of any fusion protein. For example, the transcription factors PU.1 and C/EBPα have been found to be essential factors in the differentiation of the myeloid lineage (Nutt et al., 2005; Zhang et al., 1997). The transcription factor GATA1 has been found to be essential for differentiation of the erythroid lineage (Pevny et al., 1991; Weiss et al., 1994). Mutations have been found in these factors in leukemia. Mutated transcription factors are responsible for the block in differentiation (Tenen, 2003).

Besides mutations in transcription factors, mutations have been found in genes that are involved in signal transduction. *FLT3*, a growth factor receptor, is a commonly mutated gene in AML (Gilliland et al., 2004). Due to this mutation, FLT3 is constitutively activated, also in the absence of ligand, and is able to confer a proliferate advantage to the leukemic cells.

Thus far, the most commonly mutated gene found in AML is nucleophosmin (NPM) (Falini et al., 2005). NPM is a tumor-suppressor and interacts, amongst others, with p53 (Colombo et al., 2002). In other types of cancer, p53 is the most frequently mutated oncogene (Vousden and Prives, 2005). Instead of mutations in the p53 encoding gene, certain regulators of p53 are often repressed or overexpressed in leukemia, as will be discussed further in this thesis (Chapter 4).

The recurrent mutations described for AML are given in table 1 (Mitelman et al., 2004). These mutations have the following significance in the clinic. 1) Several mutations have a prognostic value and may be used to decide on an appropriate therapy based on a more individual risk-assessment (Grimwade et al., 1998). 2) Mutations may be used as molecular tumor markers. Using techniques like quantitative PCR, minimal residual disease can be monitored (Yin and Grimwade, 2002). 3) New drugs are being developed that target mutated proteins, or the biological pathways downstream of this lesion (Deininger et al., 2003).

Some of the recurrent mutations may be found together in one leukemic clone. Other mutations never occur together. It has been inferred that combinations of mutations is excluded when the genes, in which these mutations occur, function in the same biological pathway. A cell with mutations in several genes that function in one biological pathway will not have a selection advantage. For example, the p53 pathway is important in tumor suppression. In many cancers, p53 itself is mutated. A protein like MDM2, a regulator of p53, may be affected when p53 is not mutated (see also chapter 4 of this thesis). In contrast, the PML-RARα translocation is often found in combination with a mutation in *FLT3* (Gale et al., 2005). This indicates that both mutations affect a different biological pathways.

APL

Acute promyelocytic leukemia (APL) is a subtype of AML (FAB type; AML-M3). APL is characterised by a block of differentiation in, and accumulation of promyelocytes (Sirulnik et al., 2003; Tallman, 2004). Before 1988, treatment of APL consisted of chemotherapy and resulted in long-term free survival of approximately 35% of the cases 1992; Pandolfi et al., 1992). In approximately 2% of the cases of APL the *RAR*α gene is fused to a locus called *promyelocytic leukemia zinc finger* (*PLZF*) (Chen et al., 1993).

Noteworthy of this class of patients is that

Table 1 (adepted from Jansen JH and van der Reijden BA, NTvH 2005;2;50-58)
Recurrent mutations in acute myeloid leukemia

Gene(s)	Type of mutation	Disturbed function	Cytogenetics	Prognostic value	Incidence	FAB-association
BCR ABL	Gene fusion	Signal transduction	t(9;22)(q34;q11)	-	1-2%	M0
AML1 ETO	Gene fusion	Transcription factor	t(8;21)(q22;q22)	+	10%	M2
CBFβ MYH11	Gene fusion	Transcription factor	t(16;16)(p13;q23) inv(16)(p13;q22)	+	10%	M4Eo
PML RARα	Gene fusion	Transcription factor	t(15;17)(q22;q21)	+	5-10%	M3
MLL >30 fusion genes	Gene fusion	Gene expression	t(11;…)(q23;…)	-	10%	M4/5
MLL	Internal duplication				5%	
DEK CAN	Gene fusion		t(6;9)(p23;q34)	-	1-2%	
WT-1	Point mutation and over Expression	Transcription factor		-	1-5%	
N-RAS	Point mutation	Signal transduction		-	5-10%	
FLT3-ITD	Internal duplication	Signal transduction		-	20-25%	M3
FLT3-835	Point mutation	Signal transduction			5%	
C/EBPα	Point mutation	Transcription factor		+	5%	
PU.1	Point mutation	Transcription factor			1-5%	
c-KIT	Point mutation	Signal transduction			1-5%	CBF-leukemia
P53	Point mutation	Tumor supressor			1-5%	
EVI-1 (several fusion genes)	Over expression or gene fusion	Transcription factor	t(3;...)(q26;...)	-	10%	
NPM1	Point mutation	Tumor supressor		+	35%	

they do not respond to ATRA with terminal differentiation of the cells (Guidez et al., 1994; Licht et al., 1995). Some sporadic cases have been described where the *RARα* gene is fused to either *nucleophosmin* (*NPM*) (Redner et al., 1996), *nuclear matrix-mitotic apparatus protein* (*NuMa*) (Wells et al., 1997) or the transcriptional activator *STAT5b (Arnould et al., 1999)*. The frequently found PML-RARα translocation is the main focus of this thesis.

Mouse models of APL

The PML-RARα chimeric protein contains most of the PML sequence and a large part of RARα, including its DNA and nuclear hormone binding domains. The reciprocal product, RARα-PML, is not always expressed and is believed not to be involved in transformation. The role of PML-RARα in transformation has been studied by expressing the fusion gene in immature hematopoietic cells, both in primary bone marrow cells *in vitro* as well as in transgenic animals. Expression of PML-RARα resulted in a maturation block at the promyelocytic stage and inoculation of PML-RARα transduced bone marrow cells into irradiated syngenic mice resulted in the development of retinoic acid sensitive leukemia (Grignani et al., 2000; Minucci et al., 2002). Several transgenic mouse models were generated in which PML-RARα is under the control of a myeloid promoter. Transgenic animals developed a myeloproliferative syndrome which, depending on the promoter that was used, progressed to overt leukemia in 30% to 90% of the animals after 6-12 months (Pandolfi, 2001; Pollock et al., 2001; Westervelt et al., 2003). The incomplete penetrance suggests that to develop APL, secondary mutations must occur in the transgenic animals. As mentioned before, FLT3 may be one of these secondary mutations. Introduction of an activated form of FLT3 into PML-RARα transgenic mice resulted in a decrease in latency from 8.5 months to approximately 3 months (Sohal et al., 2003). Another mutation may be the transcription factor PU.1. Some human AML samples have an acquired loss-of-function mutation in one copy of PU.1. Crossing haploinsufficient, $PU.1^{+/-}$ mice with PML-RARα expressing mice, resulted in an increase of the penetrance of APL (Walter et al., 2005).

The RARα transcription factor

ATRA, a derivative of vitamin A, can force APL cells to terminally differentiate. Paradoxically, it is the vitamin A receptor (RARα) that is mutated in APL. To understand this paradox, much research has focussed on the working mechanism of RARα. RARα belongs to the family of nuclear hormone receptors. Nuclear hormone receptors function as ligand-activated zinc-finger transcription factors (Tata, 2002). RARα must heterodimerize with another protein, RXR, to bind to the DNA. The RARα/RXR heterodimer only binds to specific sequences in the DNA; the retinoic response element (RARE). This element consists of a repeated consensus sequence (A/G)G(T/G)TCA that is separated by two or five basepairs (deThe et al., 1990b). Target genes of RARα are defined by a RARE within their promoter. In the absence of ligand, RARα/RXR represses gene transcription by recruitment of co-repressors (Alland et al., 1997; Heinzel et al., 1997; Nagy et al., 1997). Repression of gene transcription is by methylation and deacetylation (Burgers et al., 2002; Cameron et al., 1999; Jaenisch and Bird, 2003). Methylation of DNA at CpG dinucleotides and subsequent recruitment of methyl-CpG-binding proteins, and deacetylation of histones results in remodelling of the chromatin structure, making it inaccessible to the transcriptional machinery. In the presence of ligand, RARα/RXR releases the co-repressors and recruits transcriptional coactivators with histone acetyltransferase activity. Acetylation results in an 'open'

chromatin structure and renders the DNA accessible to the transcriptional machinery activating transcription (Glass and Rosenfeld, 2000; Kouzarides, 1999).

PML-RARα is able to bind to the same sequences as RARα/RXR and PML-RARα is also able to recruit the same co-repressors. However, PML-RARα does not release these co-repressors under the same conditions as RARα/RXR. PML-RARα needs much higher concentrations of ATRA to release the co-repressors and to activate gene transcription (Grignani et al., 1998; Lin et al., 1998; Di et al., 2002; Guidez et al., 1998; He et al., 1998; Melnick and Licht, 1999). Therefore, it is believed that under physiological concentrations of ATRA, PML-RARα represses gene transcription. This repression may result in the block of differentiation in APL cells. However, under pharmacological concentration of ATRA, PML-RARα will replace the co-repressors with co-activators and activate gene transcription. In this way, PML-RARα may force differentiation of APL cells.

The PLZF-RARα fusion protein can form, via its PLZF moiety, co-repressor complexes which are insensitive to ATRA (He et al., 1998). This may explain why PLZF-RARα positive cells do not differentiate in response to ATRA.

The PML protein

PML was first described as the fusion partner of RARα in the t(15;17) translocation in APL, hence the name *promyelocytic leukemia* gene (*PML*). However, PML expression is not limited to APL cells. PML is a ubiquitously expressed protein (Borden, 2002). Typically, PML is localised to the cell nucleus and is concentrated in round dots (±10 per cell, figure 3). These domains are called nuclear bodies (PML-NBs) and were first observed as early as the 1960s using electron microscopy. In APL cells the nuclear bodies are disrupted and appear dispersed into many, smaller, microspeckles (Figure 3). Treatment of APL cells with ATRA restores the localisation of PML into macrospeckles (Weis et al., 1994). Knowledge of the exact function of the PML-NBs, and of the microspeckles, remains incomplete. Over 50 protein partners, with a wide variety of functions, have been described for PML. These include chromatin remodelling factors, DNA replication factors, translation initiation factor 4E (eIF4E), SUMO-related proteins and the apoptosis related proteins p53 and MDM2 (Borden, 2002). Therefore, PML may function in transcription, DNA repair, DNA replication and mRNA transport. Noteworthy, DNA viruses start their replicative processes in PML bodies (Tang et al., 2003).

Disruption of PML-NBs through mutation of the RING-domain of PML is correlated with a loss of its growth suppression activity (Le et al., 1996), loss of apoptotic activity (Borden et al., 1998), and inability to transport certain mRNAs (Lai and Borden, 2000). Overexpression of PML in serum-starved fibroblasts induces apoptosis (Borden et al., 1997).

Mouse models have shown that the *PML* gene is not essential for survival. However, PML is essential for apoptosis induced under several types of cellular stresses (Wang et al., 1998). In addition, PML $^{-/-}$ mice are highly susceptible to infections. However, these mice develop, in a sterile environment, within 15 months, cancer with 100% penetrance (Unpublished, Pandolfi PP 4th international symposium on APL, Rome, 2005).

Not all of the PML protein is localised in the nucleus, some is localised in the cytoplasm. Cytoplasmic PML has been shown to be an essential modulator of the TGF-β signalling pathway, which is an important tumour suppressive pathway (Lin et al., 2004).

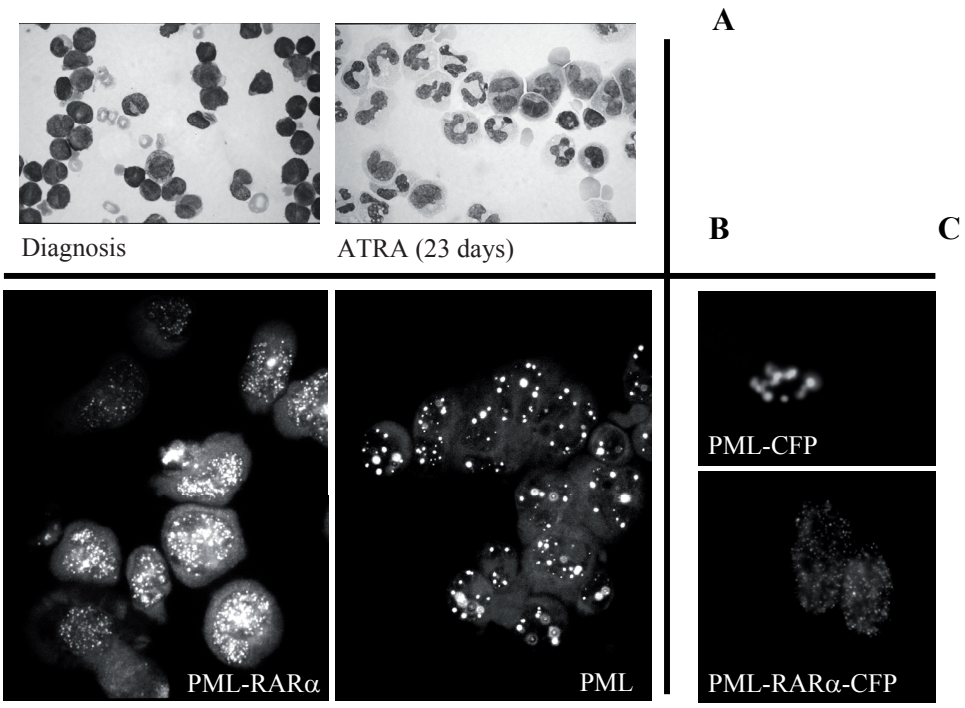

Figure 3. APL cells. (A) May-Grünwald-Giemsa staining; Cells were isolated from an APL patient at diagnosis and after 23 days of ATRA therapy (without chemotherapy). Before treatment a high percentage of promyelocytic blasts is found. During treatment, increasing amounts of maturing granulocytes are found. At day 23, many cells have both a granulocytic-like nucleus and auer rods, typical for APL cells. This indicates that the leukemic cells have differentiated (B) Immunofluorescence; APL cells, at diagnosis, and non-APL cells were stained with anti-PML specific antibody. PML is found in the nucleus of every cell and is localised in about 10 dots, called PML nuclear bodies. PML(-RARα) is dispersed in APL cells into smaller dots, called microspeckles. (C) In the upper panel PML, fused to ECFP, is expressed in HEK293 cells (1 cell is shown). In the lower panel PML-RARα, fused to ECFP, is expressed in HEK293 cells (2 cells are shown).

Mechanisms of transformation and terminal differentiation in APL

PML-RARα is the causitive oncogene in APL. Treatment with ATRA forces terminal differentiation of APL cells. Differentiation of APL cells, in response to ATRA, is accompanied by degradation of the PML-RARα oncoprotein.

Two processes may be involved in terminal differentiation of APL cells. Firstly, ATRA may activate PML-RARα-dependent gene transcription, thereby actively inducing differentiation. Secondly, degradation of PML-RARα may result in cancellation of the dominant negative effects of PML-RARα, in other words: induction of differentiation by reversal of the transforming effects. In this thesis we describe the studies we have done into the processes of transformation and differentiation (Figure 4).

PML-RARα and mechanisms of transformation

PML-RARα is able to bind to RARα target genes and is able to repress transcription of these genes. As described above, this

theory may explain some of the oncogenic properties of PML-RARα. However, several other properties of PML-RARα may add to its oncogenicity: (1) Besides disturbing RARα function, PML-RARα disturbs PML localisation and function. For example, it has been shown that the TGF-β signalling pathway is disturbed in PML-RARα expressing cells, comparable to PML -/- cells (Lin et al., 1998). (2) The p53 protein is the main executioner of apoptosis. PML-RARα expression impairs p53 stability and function (Insinga et al., 2004), making APL cells insensitive to p53-mediated apoptosis. (3) PML-RARα can bind to a larger variety of direct repeats than RARα (Kamashev et al., 2004; Jansen et al., 1995). This suggests that PML-RARα has additional targets over RARα. (4) Besides (de-)regulating transcription, PML-RARα stabilizes the kinase MNK1, at the protein level (Worch et al., 2004). Stabilization of proto-oncoproteins may contribute to the transformation of APL. (5) PML-RARα sequesters RXR to the microspeckles (Weis et al., 1994). Beside a heterodimerisation partner for RARα, RXR is a heterodimerisation partner for many nuclear hormone receptors (Chambon, 1996). PML-RARα may block the function of these other nuclear hormone receptors by sequestering RXR.

The oncogenicity of PML-RARα is dependent on certain criteria. (1) The capability to block differentiation is dependent on the presence of both the dimerization domain in the PML-part as well as the DNA binding domain in the RARα-part of PML-RARα (Grignani et al., 1996; Kastner et al., 1992; Perez et al., 1993). (2) A sumoylation site (K160) in the PML-part of PML-RARα is essential to fully develop APL (Zhu et al., 2005). This site is essential for the binding of PML-RARα to several proteins, suggesting these binding partners are needed for transformation. (3) The transforming capacity of PML-RARα depends on the presence of the myeloid specific serine protease; neutrophil elastase (Lane and Ley, 2003; Lane and Ley, 2005). This enzyme is able to cleave the PML-RARα protein. These findings may be explained by the fact that PML-RARα expression is toxic in most cell types. Low expression levels however, of PML-RARα, are probably transforming.

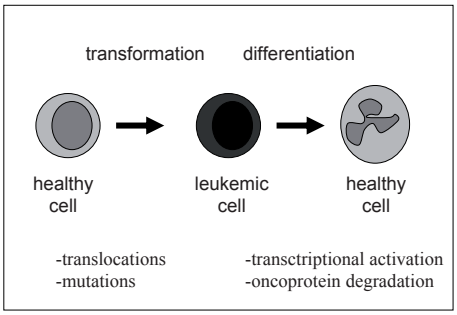

Figure 4. APL may be used to study both the process of transformation and differentiation in one disease.

PML-RARα in differentiation

Several lines of evidence support the idea that PML-RARα mediates the ATRA response in APL cells and actively induces differentiation. PML-RARα is able to bind to ATRA and activate transcription upon ATRA binding. PML-RARα increases the sensitivity to ATRA when expressed in non-APL cells and restores ATRA-sensitivity in cell lines carrying mutations in RARα (Grignani et al., 1993; Grignani et al., 1996; Ruthardt et al., 1997). PML-RARα re-expression restores ATRA-sensitivity in APL cells with constitutive degradation of the fusion protein (Fanelli et al., 1999).

Additionally, PML-RARα is degraded in APL cells after the ATRA response, a process that starts later than 12 hours after the initial exposure to ATRA. Release of the dominant negative effect on several signalling pathways may drive the APL cells into terminal differentiation (See chapter 7 “summary and perspectives” for an extensive discussion on transformation and differentiation in APL).

PML-RARα target genes

Several groups have employed microarrays to identify the genes regulated by PML-RARα and ATRA, using fresh APL cells (Meani et al., 2005), using the APL derived -PML-RARα positive- cell line NB4 (Lee et al., 2002; Liu et al., 2000; Nouzova et al., 2004; Tamayo et al., 1999; Thompson et al., 2003), or using U937 cells expressing PML-RARα under the control of a Zn^{2+} inducible promoter (Alcalay et al., 2003; Meani et al., 2005; Muller-Tidow et al., 2004; Park et al., 2003).

Ectopic expression of PML-RARα in U937 cells results in the downregulation of several genes. These genes include genes normally involved in commitment to the granulocytic lineage; *C/EBPα, JUND, GFI1, CSF3R, STAT5A, HCK, ZNF1A1*, to the erythroid lineage; *NFE2* and to the lymphoid lineage; *STAT5A, TCF3*. These targets may cause the differentiation block. Moreover, 17 DNA repair genes were found downregulated by PML-RARα. This may increase the susceptibility of APL cells to additional mutations.

Ectopic expression of PML-RARα also results in upregulation of several genes. These targets include *BCL11A, LMO1* and *JAG1*, which are implicated in self-renewal of hematopoietic stem cells. The studies revealed that PML-RARα expression may activate the NOTCH and WNT signalling pathways. Both the downregulation and the upregulation of genes by PML-RARα are counteracted by ATRA.

In U937 cells, several genes are only responsive to ATRA when PML-RARα is expressed. This suggests that these targets are not transcriptionally regulated by wild-type RARα. Treating cells with cycloheximide, an inhibitor of protein synthesis, does not inhibit the ATRA response of several of these genes. This suggests that PML-RARα directly regulates these genes. Together these data indicate that PML-RARα regulates a set of genes that are not regulated by wild-type RARα.

helix-loop-helix (HLH) proteins

Members of the bHLH family of transcription factors have been implicated in cell fate decisions in many different tissues. For example, the bHLH transcription factors MyoD and myogenin are involved in the development of muscle tissue and during neurogenesis the bHLH protein MASH-1 plays an essential role (Massar et al., 2000). As a common theme, tissue specific bHLH factors form heterodimers with members of the more ubiquitously expressed E-protein bHLH family members E12/47, HEB and E2-2, which are generally considered as promiscuous heterodimerization partners.

Inhibitors of DNA binding (ID) proteins constitute a separate class of HLH proteins lacking the basic DNA-binding domain and acting as dominant negative regulators of bHLH transcription factors by trapping them in heterodimeric complexes which are unable to bind DNA (Massar et al., 2000). In addition, ID proteins have been implicated in cell cycle control through direct interaction with the retinoblastoma protein (Norton et al., 2000), and by interaction with the centrosome (Hasskarl et al., 2004). Recent data indicate the involvement of ID proteins in angiogenesis, metastasis and tumorigenesis (de Candia et al., 2003; Fong et al., 2003; Ruzinova et al., 2003; Sikder et al., 2003). In hematopoiesis, bHLH transcription factors and their dominant negative regulators, the ID proteins, have been implicated in lymphopoiesis. Constitutive expression of ID1 targeted to pro-B cells in transgenic mice revealed impairment of B lymphocyte development (Norton et al., 1998). In addition, several mutations affecting bHLH proteins have been implicated in lymphoid leukemia. Three bHLH family members (*SCL/TAL-1*, *LYL-1* and *E2A*) are located at the sites of recurrent chromosomal translocation

breakpoints in leukemia. The t(1;14) in patients with T cell acute lymphoblastic leukemia (T-ALL) transposes the *SCL* gene to the *TCR alpha/delta* locus on chromosome 14 (Begley et al., 1989). The t(7;19) in T-ALL involves the *LYL-1* gene that is translocated to the *TCR beta* locus (Mellentin et al., 1989a). *E2A* is involved in the t(1;19) that occurs in approximately 25% of the cases of childhood pre-B ALL and results in the generation of an E2A-PBX fusion protein (Mellentin et al., 1989b).

We have found that certain HLH proteins are responsive to ATRA (Nigten et al., 2005). ATRA induced a rapid increase in ID1 and ID2, both in the APL cell line NB4 as well as in primary patient cells. In addition, a strong downregulation of E2A was observed. The simultaneous upregulation of ID1 and ID2, and the downregulation of E2A suggest a role for bHLH proteins in the induction of differentiation of APL cells following ATRA treatment. To test the relevance of this upregulation, ID1 and ID2 were overexpressed in NB4 cells. Overexpression inhibited proliferation and induced a G0/G1 accumulation. These results indicate that *ID1* and *ID2* are important retinoic acid responsive genes in APL, and suggest that the inhibition of specific bHLH transcription factor complexes may play a role in the therapeutic effect of ATRA in APL.

Gain-of-function

PML-RARα is able to act as a double dominant-negative protein, inhibiting the normal activity of both PML and RARα. Artificial RARα fusion proteins have been generated in which the PML part was replaced with a dimerizing peptide (Matsushita et al., 2006; Sternsdorf et al., 2006). In vivo, these constructs induced leukemia with very low efficiency, suggesting disruption of RARα is not sufficient for leukemogenesis. Crossing of the lines expressing the artificial RARα fusion proteins into PML null background did not result in an increased incidence of leukemia. These data suggest that PML-RARα does not simply block PML and RARα function, but rather that the fusion gene represents a gain-of-function.

Expression of PML-RARα in the myeloid cell line U937 enhances its sensitivity to the induction of differentiation by ATRA and several genes are only responsive to ATRA when PML-RARα is expressed. This suggests that also during differentiation PML-RARα may represent a gain-of-function mutation.

We investigated the mechanism by which *ID1* and *ID2* are regulated in APL cells. We found that *ID1* and *ID2* are regulated by PML-RARα through a novel mechanism, which is not shared with normal RARα, defining a gain-of-function through an as yet not recognized class of genes directly upregulated upon ATRA-induced differentiation of APL cells.

We found that the transcription factor NF-Y is involved in the gain-of-function mechanism in APL. Therefore we also investigated the function of this factor in normal hematopoiesis and we describe how NF-Y is involved in regulating the expansion of the myeloid progenitor cells. Additionally, we developed a novel protocol to investigate protein-protein interaction in a high-throughput manner using the different NF-Y protein subunits.

Furthermore we investigated the effect of PML-RARα on the p53 apoptotic pathway. We describe a novel link between PML-RARα and the p53 inhibitor MDM2 and show that MDM2 inhibitors may have therapeutic use in APL.

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Gene transactivation without direct DNA-binding defines a novel gain-of-function for PML-RARα

Abstract

PML-RARα is the causative oncogene in 5-10% of the cases of acute myeloid leukemia. At physiological concentrations of retinoic acid PML-RARα silences RARα target genes, blocking differentiation of the cells. At high concentrations of ligand, it (re)activates the transcription of target genes, forcing terminal differentiation. The study of RARα target genes that mediate this differentiation has identified several genes that are important for proliferation and differentiation control in normal and malignant hematopoietic cells. In this chapter we show that the PML-RARα fusion protein not only interferes with the transcription of regular RARα target genes. We show that the *ID1*, *ID2* and *HES1* promoters are activated by PML-RARα but, unexpectedly, not by wild type RARα/RXR. Our data support a model in which the PML-RARα fusion protein regulates a novel class of target genes by interaction with the Sp1 and NF-Y transcription factors, without directly binding to the DNA, defining a gain-of-function for the oncoprotein.

Introduction

Acute promyelocytic leukemia (APL) is characterized by an excess of immature promyelocytes in the bone marrow that fail to differentiate towards mature granulocytes. In approximately 98% of the cases, the retinoic acid receptor-α (RARα) gene is fused to the promyelocytic leukemia (PML) gene resulting in a PML-RARα fusion protein. The PML-RARα chimeric protein contains most of the PML sequence and a large part of RARα, including its DNA- and nuclear hormone-binding domains. APL blasts can be forced to terminally differentiate using pharmacological doses of all-*trans* retinoic acid (ATRA). When treated with chemotherapy, APL patients can be cured in approximately 40% of the cases. The combination of ATRA with chemotherapy leads to a remarkably high cure rate of approximately 90% [1, 2], and APL currently represents the best prognostic group amongst the different forms of leukemia. This treatment constitutes one of the first examples of successful induction of differentiation of malignant cells yielding significant clinical results.

The role of PML-RARα in transformation and terminal differentiation has been studied intensively in the past decade. PML-RARα was shown to act as a dominant oncogene, interfering with the normal function of the unrearranged PML as well as the unrearranged RARα protein. Expression of the fusion protein in immature hematopoietic cells induced a maturation block at the promyelocytic stage. Inoculation of PML-RARα-transduced bone marrow cells into irradiated syngenic mice resulted in the development of retinoic acid sensitive leukemia [3, 4]. Furthermore, PML-RARα transgenic mice developed a myeloproliferative syndrome which progressed to overt leukemia in 30% to 90% of the animals after 6-12 months, depending on the promoter that was used [5-7].

PML is a ubiquitously expressed protein that localizes to nuclear substructures termed nuclear bodies. More than 50 protein partners with various biological functions colocalize with PML [8]. In APL cells these nuclear

bodies are disrupted and dispersed into numerous small microspeckles [9]. PML has multiple tumor-suppressor functions and is involved in growth control, replicative senescence and apoptosis [10]. *PML* -/- mice are prone to develop tumors in response to various forms of stress. In addition, *PML-RARα* transgenic mice develop leukemia much faster in a *PML* -/- background [11].

Retinoic acid receptors are transcription factors that activate genes in a ligand-dependent manner. RARα binds to DNA as a heterodimer with RXR proteins. In the absence of ligand, both RARα/RXR and PML-RARα bind corepressors like N-Cor and SMRT and recruit histone deacetylases leading to gene silencing. In the presence of ligand, the corepressors are replaced by coactivators, leading to transcriptional activation. However, PML-RARα releases the corepressors at much higher concentrations of ligand compared to the unrearranged receptors. Since PML-RARα competes with unrearranged receptors for the same DNA-binding sites, the presence of the fusion protein results in dominant silencing of retinoic acid receptor target genes at physiological concentrations of ligand. At higher, supra-physiological concentrations the fusion protein can still function as a transcriptional activator releasing the corepressor complex and allowing the transcription of genes that are important for granulocytic differentiation [12-15]. The release of the differentiation block by high concentrations of retinoic acid leads to terminal granulocytic differentiation of the leukemic cells and the induction of hematological remissions in the patients. RARα target genes are not restricted to genes that contain a consensus retinoic acid receptor binding-site in their promoter. Liganded RARα is able to repress AP-1 mediated transcription [16-18]. PML-RARα abnormally regulates AP-1 activity as it stimulates AP-1-dependent transcription in the presence of ligand, whereas unliganded PML-RARα inhibits AP-1 dependent transcription [19]. In addition, RARα may interfere with GATA-2-dependent transcription. Liganded RARα enhances GATA-2-dependent gene transcription via direct protein-protein interaction [20]. This activity is retained in the PML-RARα fusion protein [21]. Furthermore, interaction of normal retinoid receptors with the transcription factor Sp1 has been shown [22, 23]. Together, the data support a model in which PML-RARα interferes with RARα target gene expression in a dominant fashion, and that restoration of target gene expression by high concentrations of ligand is important for the induction of differentiation.

Apart from interference with the function of both parental proteins in a dominant negative manner, a gain-of–function for PML-RARα is suggested by various observations. PML-RARα may bind to DNA as a heterodimer with RXR, but also independently from RXR as a homodimer [24]. It may bind to regular retinoic acid receptor binding sites consisting of a repeated consensus [(A/G)G(T/G)TCA] sequence, but the required spacing between the two half-sites is less stringent for the fusion protein. This allows the fusion protein to bind to a wider range of DNA-target sequences compared to normal receptors [25, 26]. The importance of PML-RARα as a transcriptional activator of differentiation-inducing genes was shown by in vitro experiments. Expression of PML-RARα in the myeloid cell line U937 (that also expresses normal retinoic acid receptors) enhanced their sensitivity to the induction of differentiation by ATRA [27]. In addition, forced expression of PML-RARα, but not RARα, in an ATRA resistant APL cell line with constitutive degradation of the chimeric protein restored ATRA sensitivity [28]. Importantly, in APL patients that became resistant to differentiation induction with ATRA during therapy, additional mutations were found in the ligand-binding domain of the RARα part of the PML-RARα fusion protein, indicating an important role for

the fusion protein during the retinoic acid-induced differentiation of the leukemic cells [29]. Finally, experimental mouse models in which various natural and artificial RARα fusion proteins were expressed also support a model in which interference with the function of RARα and PML is important, but in addition, they suggest that the PML-RARα fusion protein exhibits gain-of function characteristics, unique to the fusion protein [7, 30]. So far, the mechanisms behind these observations remain largely unclear.

We have previously shown that the transcription factor inhibitors ID1 and ID2 are upregulated upon treatment with ATRA and play a role in cell cycle arrest during APL differentiation [31]. In this study we investigated the mechanism by which these genes are regulated in APL cells. We found that *ID1* and *ID2* are regulated by PML-RARα through a novel mechanism, which is not shared with normal RARα, defining an as yet unrecognized class of retinoic acid-induced genes in APL.

Materials and Methods

Cell culture

NB4 cells, U937 and U937-PR9 cells (kindly provided by Dr. P.G. Pelicci and Dr. F. Grignani) were cultured in RPMI 1640 medium (Gibco, Gaithersburg, MD), Hep3B cells in IMDM (Gibco) and HEK293 cells in DMEM + 2 mM L-glutamine. Media were supplemented with 10% FCS (Gibco). ATRA was used at a final concentration of 10^{-6} M (Sigma, St Louis, MO) and $ZnSO_4$ at 100μM. Cycloheximide (ICN, Costa Mesa, CA) was added 30 min. prior to ATRA at 4 μg/ml.

DNA constructs and antibodies

The human *ID1* promoter was obtained from J. Campisi (University of California, USA). The *ID2* promoter [32] was cloned into the XhoI-HindIII sites of the pGL3-basic vector (Promega, Madison WI, USA) after amplification with the following primers: sense 5'-GTACGGTACCTCGAGTTGGGCATGGT TTGCAATA, and anti-sense 5'-GTACAGA TCTAAGCTTGAAGCCCGAGCCCGGC. The *HES1* promoter (containing the –252 to +31 promoter fragment of *HES1*) was cloned into the XhoI-HindIII site of the pGL3-basic vector (Promega) after amplification with the following primers: sense 5'- GTACCTCGA GGAGAGTAGCAAAGGGTTAAAATCCT, anti-sense 5'-GTACAAGCTTCTGTTATC AGCACCAGCTCCG. $RARE_3$-tk-luc [33], PML-RARαΔR [34] and PML-RARαΔCC, PML-RARα, RARα and RXR expression constructs [25] were as described. FLAG-PML-RARα was from A. Tomita [35] and was recloned into a CMV-expression vector. *ID1* promoter deletion/mutation fragments were constructed by PCR and cloned into the XhoI-HindIII sides of PGL3-basic (Promega). All constructs were sequence verified. The dominant negative NF-YA (YAm29) construct and anti-NF-YB polyclonal antiserum were from R. Mantovani (University of Milan, Italy). pGEX- Sp1 was from H. Rotheneder (University of Vienna, Austria). Anti-Sp1 (PEP-2), anti-NF-YA (H-209), anti-RARα (C-20) and anti-ID1 (Z-8) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-PML rabbit antibody was as described [9]. Anti-FLAG (M2) antibody was from Sigma. Anti-murine HES1 rabbit polyclonal antibody was a kind gift from Dr. Tetsuo Sudo, Toray Industries, Inc., Japan. As a control in the ChIP experiments, human IgG was used (Ivegam, Sanquin, The Netherlands).

Northern blotting

Total cellular RNA was isolated by guanidium-isothiocyanate lysis and centrifugation on a 5.7 M cesium chloride cushion. 10μg of total RNA was size-fractionated by 1% agarose-formaldehyde gel electrophoresis and transferred onto Hybond N^+ nylon membranes (Amersham, Buckinghamshire, UK). Filters

were hybridized at 65°C overnight in phosphate buffer (2N NaH_2PO_4, 7% sodium dodecyl sulphate (SDS), 1 mM EDTA pH 8 and 1% BSA). DNA probes were labeled with [^{32}P]α-dATP by random primed labeling (Boehringer, Mannheim, Germany). After hybridization, filters were washed in 0.2xSSC/0.2% SDS for 15 minutes at 65°C. Northern blots were hybridized to radiolabeled human *ID1* or *ID2* probes (kindly provided by Dr. S. Stegman). As a control for equal loading, filters were stripped and hybridized to a 777bp HindIII-EcoRI human GAPDH fragment.

Transactivation studies

Cells were transfected using calcium-phosphate precipitates with 0.25 μg pGL3-*ID1* or -*ID2* promoter, 0.05 μg nuclear receptor expression vector, 0.1 μg of Renilla vector (pRL-CMV, Promega) and 1 μg YAm29 expression vector. The total amount of DNA was normalized to 1.4 μg for all transfections using empty vectors. Cells were harvested 16 hours after ATRA treatment using 100μl Passive Lysis Buffer (Promega). Firefly luciferase and Renilla luciferase activities were measured on a luminometer (Lumat LB 9507, Perkin-Elmer/Applied Biosystems, CA, USA) using Dual-Luciferase Reporter Assay System reagents (Promega).

Electrophoretic mobility shift assay (EMSA)

Nuclear extracts were prepared as described [36]. 5 μg was incubated in a total volume of 15 μl containing 1 μg of double stranded poly(dI)-poly(dC), 10mM Hepes, 50mM KCL, 1 mM DTT, 1 mM PMSF, 2.5 mM $MgCl_2$, 10% (w/v) glycerol, 300 μg/ml BSA and 0.5 ng of a labeled double stranded oligonucleotide probe: *ID1* 5'-CCGCCCATTGGCTGCTTTTGAACGT. To show specificity of binding, non-labeled 100 fold excess of double-stranded oligonucleotides were added to compete for binding with the labeled probe. For this, either the *ID1* probe (self competition), a sequence not containing any CCAAT box 5'-TCAGAGTTCAAGGTTCTAGTCGCTGC GGC, or a NF-Y binding site containing oligonucleotide from the CD10 gene was used 5'-ATCCCGACCAATGAGCGCACGGGGCC GGGT [37]. DNA-protein complexes were resolved on a 5% non-denaturing polyacrylamide gel in 0.5xTBE buffer.

GST pull-down

GST-fusion proteins were produced in E.coli BL21 which were induced at A_{600} = 0.5 with 300 μM IPTG for 4 hours. Proteins were released by sonication and loaded onto glutathion-agarose (Sigma) by incubation at 4ºC for 2 hours in lysis buffer (0.15 M NaCl, 50 mM Tris [pH 8.3], 10 mM EDTA, 0.5% NP40 and protease inhibitors). Pull-down was performed using *in vitro* translated PML-RARα and PML-RARαΔcc (reticulocyte lysate, Promega) or rHSp1 (Promega), by 2 hours incubation at 4°C. Beads were washed in lysis buffer and subsequently resuspended in SDS-loading buffer.

Chromatin immunoprecipitation (ChIP)

DNA-protein cross-linking was done for 30 min at room temperature by adding formaldehyde at a final concentration of 1% directly to the culture medium. Cross-linking was stopped by the addition of glycine to a final concentration of 125 mM. Cells were washed with ice-cold phosphate-buffered saline, buffer B (10 mM EDTA, 0.5 mM EGTA, 0.25% Triton X-100, 20 mM HEPES [pH 7.6]), buffer C (1 mM EDTA, 0.5 mM EGTA, 0.15 M NaCl, 50 mM HEPES [pH 7.6]) and resuspended in incubation buffer (0.15% SDS, 1% Triton X-100, 0.15 M NaCl, 1 mM EDTA, 0.5 mM EGTA, 20 mM HEPES [pH 7.6] and protease inhibitors) at 33x10^6 cells/ml. Chromatin was sonicated using the Bioruptor (Cosmo Bio, Tokyo, Japan), at high intensity for 15 min with 0.5 min. intervals. Insoluble

material was removed by centrifugation at 4°C for 15 min. 120 µl supernatant was incubated with 30 µl precoated protein A/G plus agarose beads 50% v/v (SantaCruz), 0.1% BSA, 36 µl 5x incubation buffer, protease inhibitors and 2-5 µg antibody (anti-NF-YA, anti-PML, anti-FLAG or non-specific IgG from human serum) and rocked at 4°C for 16 hours. Beads were harvested by centrifugation and washed twice with buffer 1 (0.1% SDS, 0.1% NaDOC, 1% Triton X-100, 0.15 M NaCl, 1 mM EDTA, 0.5 mM EGTA and 20 mM HEPES [pH 7.6]), once with buffer 2 (0.1% SDS, 0.1% NaDOC, 1% Triton X-100, 0.5 M NaCl, 1 mM EDTA, 0.5 mM EGTA and 20 mM HEPES [pH 7.6]), once with buffer 3 (0.25 M LiCL, 0.5% NaDOC, 0.5% NP-40, 1 mM EDTA, 0.5 mM EGTA, 20 mM HEPES [pH 7.6]), and twice with buffer 4 (1 mM EDTA, 0.5 mM EGTA, 20 mM HEPES [pH 7.6]). Chromatin antibody complexes were eluted by the addition of 1% SDS and 0.1 M NaHCO3 to the pellet and incubated for 20 min. at room temperature. Cross-linking was reversed by the addition of NaCl (0.44 M final conc.) and incubation of the eluted samples for at least 4 h. at 65°C. DNA was recovered by phenol-chloroform-isoamylalcohol extraction followed by chloroform-isoamylalcohol extraction and precipitated by the addition of 0.1 volume of 1 M sodium acetate (pH 5.2), and 2.5 volumes of ethanol. Precipitated DNA was dissolved in water, and input as well as immunoprecipitated DNA was analyzed using quantitative PCR for genomic sequences from the *ID1, ID2, RAR*β *and p21* promoter regions [38].

Quantitative PCR

Quantitative PCR to measure ID1, ID2 and HES1 mRNA expression was performed with the ABI/PRISM 7700 Sequence Detection system (ABI/PE, Foster City, Ca, USA). As a reference gene *PBGD* was used. The primer/probe sequences for the *ID1* gene were:
sense 5'-
GTTACTCACGCCTCAAGGAGCT,
anti-sense 5'-
GAGAATCTCCACCTTGCTCACC,
probe FAM 5'-
CCCACCCTGCCCCAGAACCG;
for *ID2*: sense 5'-
GACTGCTACTCCAAGCTCAAGGA,
anti-sense 5'-
CGTGCTGCAGGATTTCCAT,
probe FAM 5'-
CCCAGCATCCCCCAGAACAAGAAGG;
for HES1: sense 5'-
GAAGGCGGACATTCTGGAAA,
anti-sense 5'-
GCGGGTCACCTCGTTCAT.
PCRs were done in a 50µl reaction mixture (1.25 U AmpliTaq Gold, 1xbuffer A (both Perkin-Elmer), 250 mM dNTPs (Pharmacia) and 5 mM $MgCl_2$ for 10 min. at 95°C followed by 45 cycles of 15 sec. at 95°C and 1 min. at 62°C. Quantitative PCR following ChIP was done using Sybr Green PCR (Applied Biosystems). Primer sequences for the *ID1* promoter were;
sense 5'-
CACTGCGAGCAGGCACTAGAC,
anti-sense 5'-
AGCCACAGCTTGTCTTT;
for the *ID2* promoter; sense 5'-
CTGTACTCTATTTACCACCCCAGCTG
and anti-sense 5'-
GGCGTGGGCTTGGTTCTT;
for the *RAR*β promoter; sense 5'-
TTGGGTCATTTGAAGGTTAGCA,
anti-sense 5'-
CACACAGAATGAAAGATTGAATTGC,
for the *p21* gene sense 5'-
GGCGGGGCGGTTGTAT
and anti-sense 5'-
AAGGAACTGACTTCGGCAGC.
for the *HES1* promoter; sense 5'-
AGTCAAAGCAGCTCTGTTACATATGAG,
anti-sense 5'-
TTTGGTCTTGATCTTGTCTATTTCTTTT.

Results

ID1 and *ID2* are direct retinoic acid-responsive genes in APL cells

Basic helix-loop-helix (bHLH) transcription factors and their inhibitors, ID proteins, play crucial roles in the regulation of differentiation in various cell types. We have shown that ID1 and ID2 are upregulated in APL cells upon exposure to ATRA [31]. Upregulation of ID1 and ID2 mRNA was confirmed with qPCR (Figure 1A and B), showing clear upregulation within 4 hours after the addition of ATRA. To investigate whether ID1 and ID2 were directly upregulated by ATRA, cells were treated with cycloheximide prior to the addition of ATRA to inhibit protein translation. Both ID1 and ID2 mRNAs were upregulated within 0.5 hours by ATRA, regardless of the addition of cycloheximide (Figure 1C and D). This indicates that both genes were directly upregulated, without intermediate protein production.

The *ID1* upstream promoter is transactivated by PML-RARα but not by RARα/RXR

To identify the regulatory DNA sequences through which the induction of *ID1* by retinoic acid was mediated, we analyzed the 5' upstream promoter sequence in transactivation assays. A 963 bp fragment of the promoter, including the putative TATA box, was cloned into a luciferase reporter construct. When expressed alone, RARα, RXR and PML were not able to transactivate the *ID1* promoter both in the absence and presence of ATRA (Figure 2A). In contrast, PML-RARα transactivated the promoter more than 12-fold in an ATRA-dependent fashion. Surprisingly, the combination of RARα/RXR

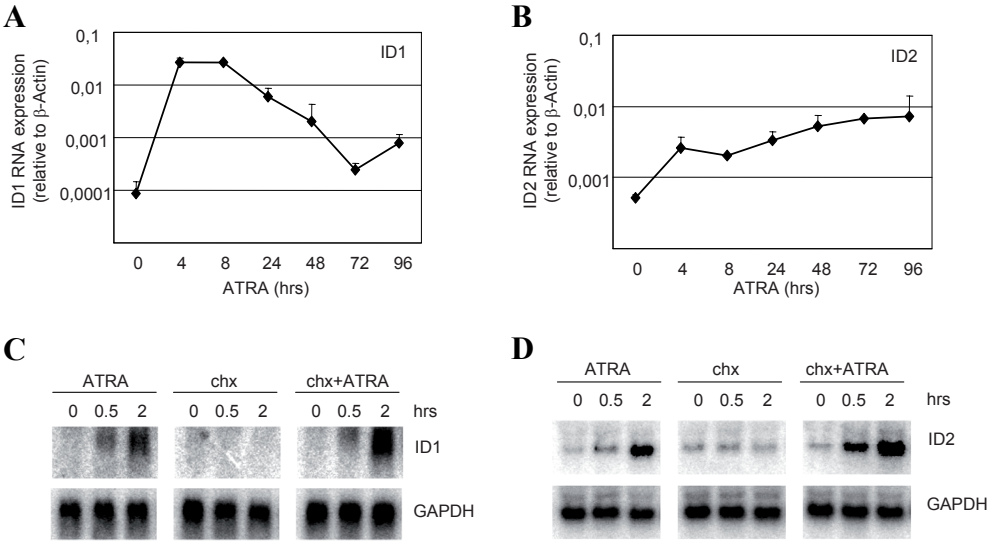

Figure 1. *ID1* and *ID2* are direct retinoic acid target genes in NB4 cells. Quantitative PCR and Northern blot analysis of NB4 cells treated with ATRA. mRNA was isolated from NB4 cells and ID1 **(A)** and ID2 expression **(B)** were determined using specific primers and probes by quantitative PCR (n=4). Quantities were normalized based on β-actin expression. To investigate whether the induction of *ID1* and *ID2* was dependent on intermediate protein production, cells were treated with cycloheximide alone (4μg/ml) or with the combination of cycloheximide and ATRA (10^{-6} M). Blots were hybridized using radiolabeled *ID1*- **(C)** and *ID2*-specific **(D)** probes. As a control for equal loading blots were stripped and rehybridized with GAPDH specific probes.

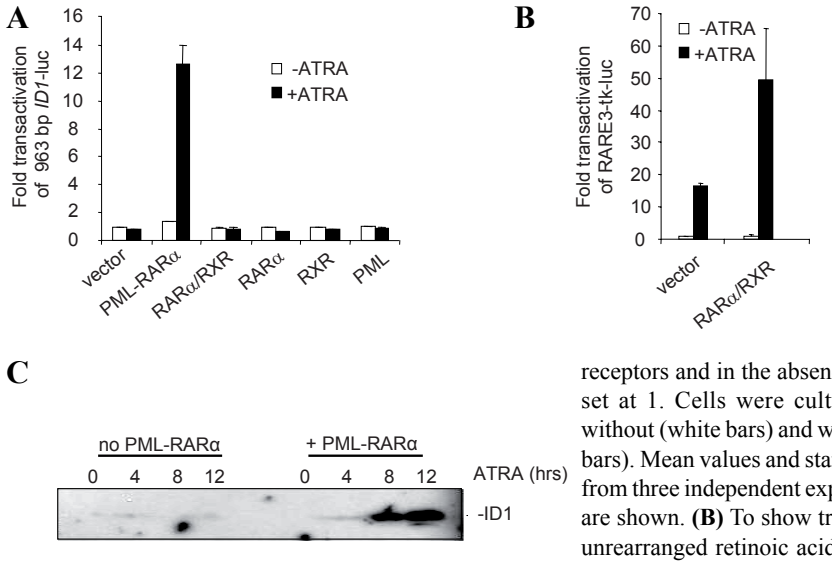

Figure 2. The ID1 promoter is transactivated by PML-RARα but not by RARα/RXR. (A) Hep3B cells were transfected with the 963bp ID1 promoter-luciferase reporter construct (ID1-luc) together with a control vector expressing Renilla-luciferase. In addition, vectors coding for the various proteins indicated in the figure were transfected. Transactivation is expressed as arbitrary units and is corrected for transfection efficiency measured by Renilla luciferase. Background luminescence of the cells transfected with only the reporter construct without nuclear receptors and in the absence of ATRA was set at 1. Cells were cultured for 16 hrs without (white bars) and with ATRA (black bars). Mean values and standard deviations from three independent experiments +/- SD are shown. **(B)** To show transactivation by unrearranged retinoic acid receptors, cells were transfected with the RARE3-tk-luc vector, containing three bona-fide RAREs. Mean values of three independent experiments +/- SD are shown. **(C)** ID1 expression is increased by PML-RARα in the presence of ATRA. The zinc-inducible PML-RARα cell line U937-PR9 was grown in the absence (left panel) or presence (right panel) of zinc for 16 hours. Cells were treated with ATRA and harvested at the indicated time points. Proteins were size-fractionated by SDS-PAGE. Immunostaining was done with anti-ID1 antibody.

did not transactivate the promoter, both in the absence and presence of ATRA. To verify that RARα and RXR were expressed and functional, a control luciferase construct was used containing three bona-fide RAREs from the RARβ promoter ($RARE_3$-tk-luc [39]). This construct was strongly transactivated in the presence of ATRA through endogenous retinoic acid receptors (Figure 2B, left bars). This was further enhanced when RARα and RXR were co-transfected (2B, right bars). Together this shows that PML-RARα may regulate *ID1* expression through the upstream 963 bp promoter whereas RARα/RXR cannot.

To test whether PML-RARα expression would result in ATRA-dependent induction of the endogenous *ID1* gene, we used the U937-PR9 cell line that is stably transfected with a Zn^{2+}-inducible PML-RARα expression cassette [34]. In PML-RARα expressing U937 cells, ATRA strongly induced ID1 expression (Figure 2C, right panel), in contrast to U937 cells that did not express PML-RARα (2C, left panel).

Transactivation of the *ID1* promoter is dependent on GC- and CCAAT box motifs

To determine the DNA sequences that are relevant for the observed PML-RARα-dependent transactivation, we analyzed

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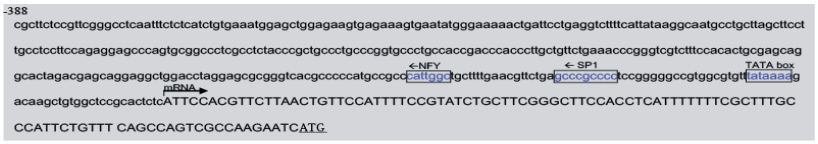

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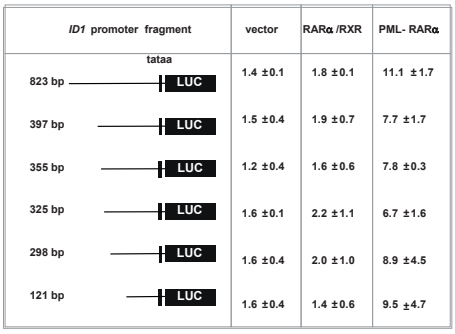

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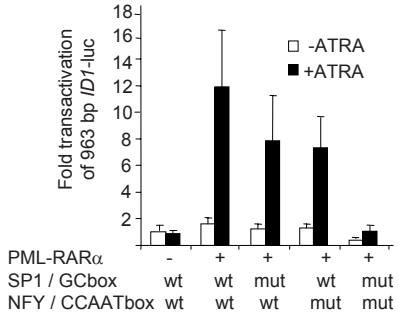


Figure 3. The CCAAT- and GC-box in the *ID1* promoter are required for PML-RARα mediated transactivation. (A) *ID1* promoter region showing the presence of consensus Sp1 and NF-Y binding sites. **(B)** Transactivation of *ID1* promoter-luciferase constructs. Several deletion constructs were generated and transfected in combination with RARα plus RXR, or with PML-RARα. Transactivation assays were performed as described in figure 2. Mean values from three independent experiments +/- SD are shown. **(C)** To investigate the importance of the putative Sp1 and NF-Y binding sites in the -121 bp upstream promoter sequence of the *ID1* gene for transactivation by PML-RARα, these sites were mutated in the context of the –963 bp promoter fragment. Mutations were introduced either alone, or in combination. Transactivation by PML-RARα was performed as described in figure 1. Sequences were mutated as follows; GC box: CCGCCC was replaced by CTATCC and for the NF-Y site: ATTGG was replaced by ACACG.

the 963 bp upstream promoter construct of the *ID1* gene further. The consensus RAR/RXR binding sequence (RARE) consists of a repeated (A/G)G(T/G)TCA sequence that is separated by two or five base pairs. However, in the 963 bp *ID1* promoter, no consensus RARE was found. As PML-RARα may bind to a much wider variety of DNA sequences [25, 26], we made luciferase constructs with different promoter truncations. All the truncation mutants could still be transactivated by PML-RARα (Figure 3B), including the smallest, 121bp promoter construct. This construct does not contain any sequence that even remotely resembles a retinoic acid receptor-binding site (Figure 3A). Analysis of the 121 bp promoter sequence for putative binding sites for other transcription factors showed a perfect consensus-binding site for the transcription factors NF-Y (CCAAT box), and Sp1 (GC box). To test their relevance, we mutated these sites individually and in combination in the context of the 963 bp promoter fragment. Mutation of the GC box or the CCAAT box alone partially impaired transactivation by PML-RARα, while deletion of the GC box and the CCAAT box in combination almost completely abolished transactivation (Figure 3C, raw Renilla and Firefly luciferase data are

given in supplemental figure 1, showing that normalization using Renilla did not change the results in a qualitative manner). This indicated that transactivation of the *ID1* promoter by PML-RARα was dependent on the GC and CCAAT motifs.

Functional NF-Y is necessary for transactivation of the *ID1* promoter by PML-RARα

Sp1 and NF-Y are ubiquitously expressed transcription factors. NF-Y is a trimeric protein complex consisting of the subunits NF-YA, NF-YB and NF-YC. All three subunits are necessary for the complex to bind to the DNA [40]. Using real-time PCR, we confirmed that Sp1 and all three NF-Y subunits were expressed in primary APL cells (supplemental figure 2). Binding of Sp1 to the promoter of *ID1* was shown previously [41]. Therefore, we tested whether NF-Y was able to bind to the CCAAT site from the *ID1* promoter. Incubation of a radioactively labeled DNA-probe containing the CCAAT box with cellular protein extracts resulted in a clear shifted complex (Figure 4A, lane 1). This shift was competed by a 100-fold excess of non-labeled probe (lane 2) and by an excess of an oligo containing the NF-Y binding site of the CD10 promoter (lane 5 [42]), but not by an excess of probe lacking a NF-Y binding site (lane 3). When anti-NF-YB antiserum was added (lane 4) the protein-DNA complex was supershifted, identifying the DNA binding protein complex as NF-Y. To further test whether NF-Y is present on the endogenous *ID1* promoter, we performed chromatin immunoprecipitation (ChIP) assays. Using anti-NF-YA antibody, recovery of the *ID1* promoter sequences from U937 cells was over 70 times higher than recovery with non-specific IgGs (Figure 4B, right bars). In the same experiment, no enrichment was seen for the *RAR*β promoter (Fig. 4B, left bars), indicating that NF-Y is present on the endogenous *ID1* promoter but not on the *RAR*β promoter.

A dominant-negative form of NF-YA (YAm29) has been described [43]. This mutated NF-YA subunit can still bind to the NF-YB and NF-YC subunits, but is not able to bind to DNA, preventing the formation of a functional trimeric NF-Y complex. To investigate whether functional NF-Y was necessary for the transactivation of *ID1*, the YAm29 mutant was tested in a transactivation assay. Transactivation of *ID1* by PML-RARα upon ATRA treatment was severely decreased in the presence of dominant-negative NF-YA (Figure 4C). This effect was promoter-specific as YAm29 did not influence transactivation of the *RAR*β promoter construct ($RARE_3$-tk-luc) by PML-RARα or RARα/RXR (data not shown).

Upregulation of *ID1* is dependent on PML-RARα, but does not require the DNA-binding domain of the fusion protein

To test whether PML-RARα might transactivate the *ID1* promoter without directly binding to the DNA, we used a PML-RARα construct from which the DNA binding domain was deleted (PML-RAR/ΔR, [34]. PML-RAR/ΔR has been shown to be unable to transactivate a promoter containing a bona-fide RARE. In contrast, PML-RAR/ΔR retained the ability to transactivate the *ID1* promoter (Figure 4D). This indicated that transactivation of the *ID1* promoter by PML-RARα does not require direct binding of PML-RARα to the DNA.

PLZF-RARα is generated by a t(11;17) translocation that is found in approximately 2% of the patients with APL. It contains the same part of the retinoic acid-receptor as PML-RARα. Similar to PML-RARα, this fusion protein may form homodimers with DNA-binding capacity [44]. Interestingly, PLZF-RARα was not able to transactivate the *ID1* promoter, suggesting that the PML part of PML-RARα is necessary for transactivation of the *ID1* promoter (Figure 4D). When

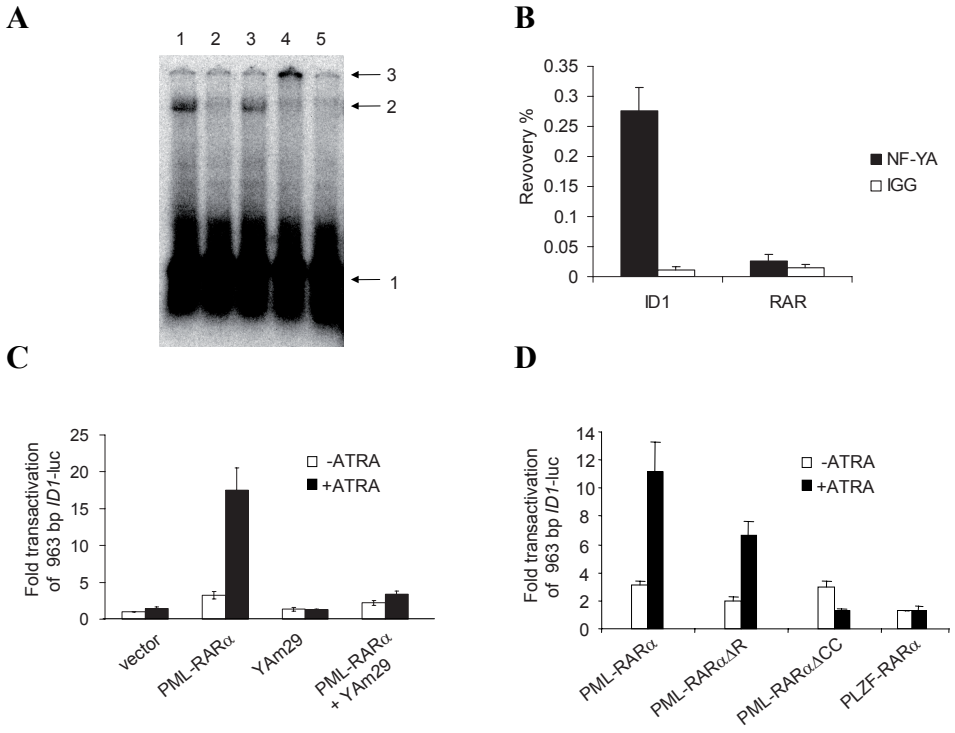


Figure 4. NF-Y binds to the CCAAT-box element from the *ID1* promoter, and is required for PML-RARα mediated transactivation. (A) EMSA showing binding of NF-Y to the putative NF-Y binding site from the *ID1* promoter. Hep3B-nuclear extracts were incubated with a labeled DNA probe containing the NF-Y-box from the *ID1* promoter region. A clearly shifted protein-DNA complex was seen (lane 1). Competition experiments were done with 100x cold ID1 probe (lane 2) and with an unlabeled probe containing the confirmed NF-Y binding site from the CD10 gene promoter (lane 5). In addition, a sequence without any recognizable NF-Y binding site was used for competition (lane 3). Addition of anti-NF-YB antibody shifted the complex to a higher molecular weight complex (lane 4). Arrows indicate free probe (1), shifted DNA-protein complex (2) and the supershifted DNA-protein-antibody complex (3). **(B)** To detect DNA-binding of NF-Y in intact cells, ChIP assays were performed using anti NF-YA antibodies. As a control, the non-specific IgG fraction from human serum was used. The Y axis shows the recovery (%) of *ID1* or *RAR*β sequences relative to the input in U937 cells, NF-Y clearly bound to the *ID1* promoter (right bars), but not to the *RAR*β gene (left bars). Mean values from three independent experiments +/- SD are shown. **(C)** To further show the importance of NF-Y for the PML-RARα mediated transcriptional activation of *ID1*, transfections (as described in figure 2) were performed using the dominant-negative NF-YA subunit (Yam29) and the 963 ID1 promoter construct. In the presence of YAm29 PML-RARα mediated transcription was severely diminished (right bars). **(D)** To investigate the importance of different domains of PML-RARα, vectors encoding the DNA binding-defective PML-RARαΔR mutant, and a mutant lacking the coiled-coil protein-protein interaction domain of PML (PML-RARαΔCC) were used. In addition, a PLZF-RARα expression construct was used. Transfections were performed as in figure 2 using the 963 *ID1* promoter construct, mean values of three independent experiments +/- SD are shown.

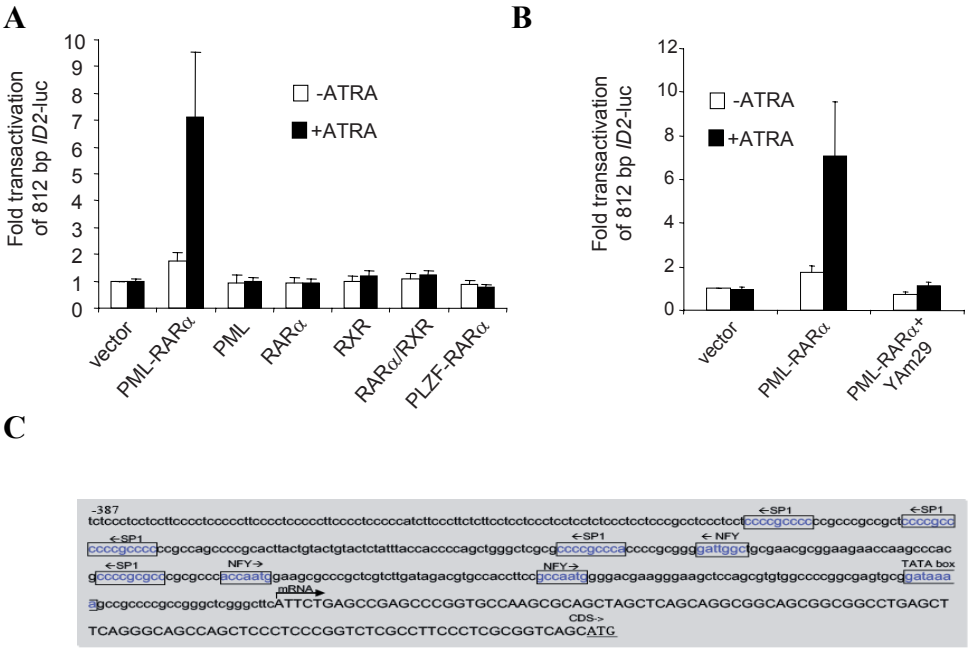


Figure 5. The *ID2* promoter is transactivated by PML-RARα but not by RARα/RXR. (A) Cells were transiently transfected with a 812 bp *ID2*-luciferase reporter construct (*ID2-luc*) . Transfections and controls were as described in figure 2. Background luminescence of the cells transfected with only a reporter construct (no nuclear receptor and no ATRA) was set at 1. Transactivation was measured after treatment without (white bars) and with (black bars) 10^{-6}M ATRA. Mean values from at least three independent experiments +/- SD are shown. **(B)** Dominant negative NF-Y (Yam29) inhibits the transactivation of the ID2 promoter construct by PML-RARα. Transfections were done as described in figure 2, mean values of three independent experiments +/- SD are shown. **(C)** Promoter region of the *ID2* gene with putative Sp1 and NFY binding sites.

the coiled-coil domain of PML-RARα was deleted (PML-RAR/ΔCC [25, 34]) it was no longer able to transactivate the *ID1* promoter (Figure 4D). As this domain is involved in protein-protein interactions, this suggested that PML-RARα homodimerization or interaction of PML-RARα with another protein was necessary for the transactivation of *ID1*.

The *ID2* promoter is also transactivated by PML-RARα but not by RARα/RXR

To investigate whether *ID2* was regulated in a similar manner as *ID1*, we cloned the upstream promoter of *ID2* (812bp) into a luciferase reporter plasmid. Similar to *ID1*, no consensus retinoic acid receptor-binding site could be found in the *ID2* promoter sequence. Also comparable to *ID1*, RARα, RXR, PML, PLZF-RARα and RARα/RXR did not transactivate the *ID2* promoter whereas PML-RARα did (Figure 5A). Furthermore, transactivation of *ID2* by PML-RARα was abolished in the presence of dominant-negative NF-YA (Figure 5B). Inspection of the *ID2* promoter sequence revealed 3 NF-Y and 5 Sp1 consensus binding sites (Figure 5C). Together, these data show that like *ID*1, *ID2* may also be transactivated by PML-RARα without direct DNA binding of the fusion protein.

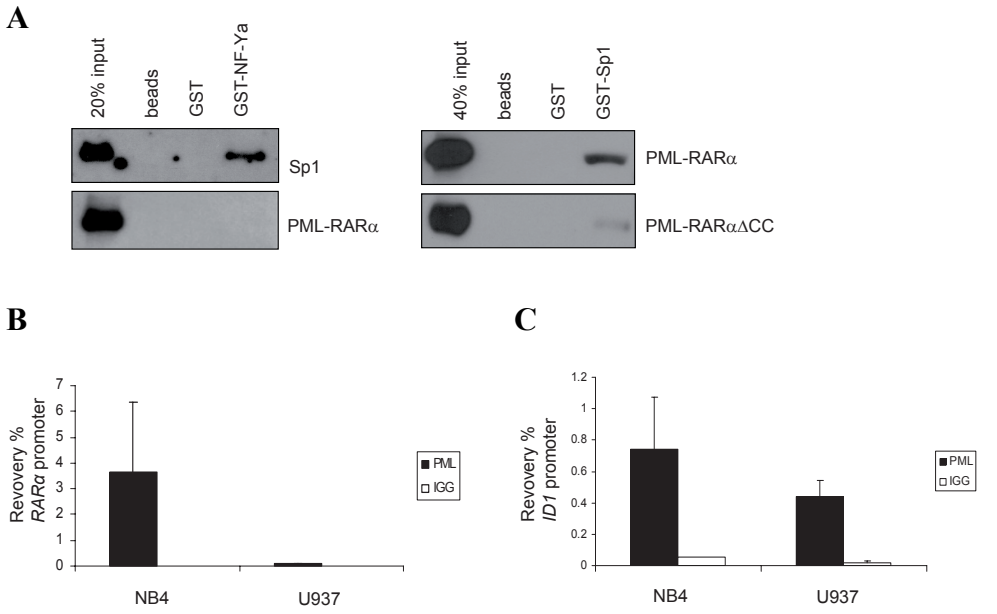


Figure 6. PML-RARα binds Sp1 and binds to the endogenous ID1 promoter. (A) To test binding of PML-RARα to Sp1 and NF-Y, GST pull-down experiments were performed. Empty beads and beads loaded with GST or GST-NF-YA (panels to the left) were incubated with in vitro translated Sp1 (positive control) or in vitro translated PML-RARα. GST-Sp1 (panels to the right) was incubated with in vitro translated PML-RARα or PML-RARαΔCC. Beads were washed and subsequently resuspended in loading buffer. Protein was resolved on SDS-PAGE. Immunostaining was performed with anti-Sp1 or anti-RARα antibody. Clear interactions between NF-YA and Sp1 and between PML-RARα and Sp1 were observed. To show binding of PML-RARα to the endogenous RARβ **(B)** and ID1 **(C)** genes, ChIP assays were performed in PML-RARα positive NB4 cells and in PML-RARα negative U937 cells. Cells were treated with ATRA for 30 min. ChIP was done with anti-PML antiserum. As a control, the non-specific IgG fraction from human serum was used. The Y axis shows the recovery (%) of ID1 or RARβ sequences relative to the input. Mean values of 4 independent experiments are shown.

PML-RARα directly interacts with Sp1

NF-Y and Sp1 have been shown to work in concert on many promoters. Direct physical interaction between Sp1 and NF-Y has been shown, indicating that these proteins may bind to adjacent DNA binding sites and form a complex that regulates transcription [45]. We investigated whether PML-RARα could physically interact with Sp1 or NF-YA. GST-tagged, bacterially produced NF-YA, and Sp1 proteins were made, as well as in vitro translated (reticulocyte lysate) PML-RARα for GST pull-down experiments. rhSp1 was commercially available. Whereas a clear interaction was observed between GST-NF-YA and recombinant Sp1 (Figure 6A), in none of the conditions tested we could show a direct interaction between NF-YA and PML-RARα. In contrast, an interaction between PML-RARα and Sp1 was readily observed, whereas no interaction was seen with non-loaded or GST-loaded beads (Figure 6A). GST-Sp1 was also able to capture PML-RAR/ΔCC although with a much lower efficiency than PML-RARα. This suggests that the binding of PML-RARα is partly through the coiled-coil

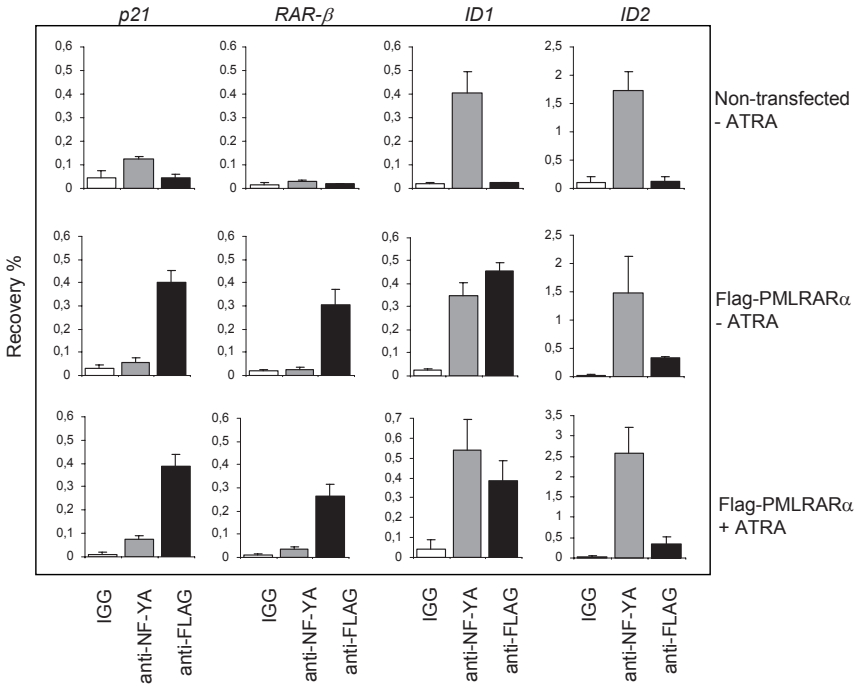


Figure 7. Binding of PML-RARα to the endogenous *p21, RAR*-β, *ID1* and *ID2* genes. To show binding of PML-RARα and NF-Y to the endogenous *p21, RAR*-β, *ID1* and *ID2* genes ChIP assays were performed. As anti-PML antibodies do not discriminate between the unrearranged PML protein and the PML-RARα fusion protein, FLAG-tagged PML-RARα was used. Cells were transfected with or without FLAG-PML-RARα and cultured in the presence or absence of 10^{-6} M ATRA. ChIP was performed using anti-FLAG-antibodies, anti-NF-YA antibodies and control IgGs. The Y axis shows the recovery (%) of *ID1/ID2/p21* or *RAR*β sequences relative to the input. Mean values of three independent experiments +/- SD are shown.

region and partly through the RARα part of the fusion protein. This is in agreement with earlier publications that have shown binding of both PML and RARα to Sp1 [22, 23].

PML and PML-RARα are present on the endogenous *ID1* promoter

To show the physical interaction of PML-RARα with the endogenous *ID1* and *ID2* promoters in intact cells, we performed ChIP assays using PML-specific antibodies. The *RAR*β promoter, that contains a well defined RARE, served as a positive control. In NB4 cells, we found a clear enrichment for the *RAR*β promoter (Figure 6B, right panel). In contrast, in U937 cells which are PML-RARα negative, no enrichment for the *RAR*β promoter was seen (6B, left panel), indicating that PML-RARα binds to the *RAR*β promoter in NB4 cells. The *ID1* gene was also precipitated with anti-PML antibody in NB4 cells (Figure 6C, right panel). However, this was not only observed in the PML-RARα positive NB4 cells, but also in the PML-RARα negative U937 cells (Figure 6C, left panel). This indicates that in U937 cells, unrearranged PML protein was present on the *ID1* promoter. As the anti-PML does not discriminate between PML and PML-RARα, we could not determine which of these two

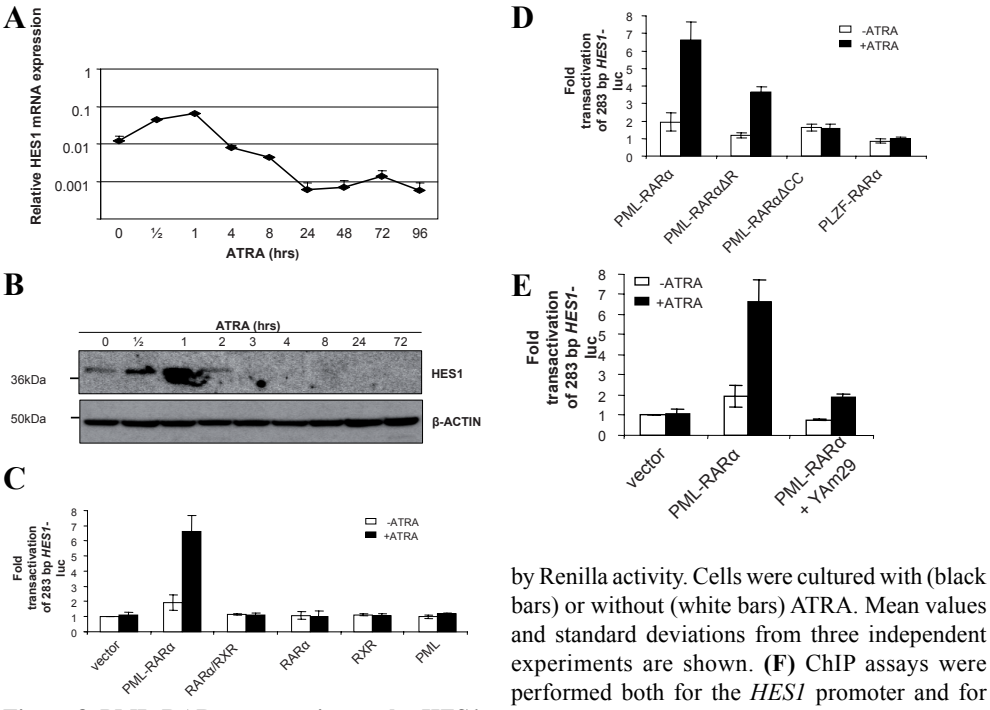
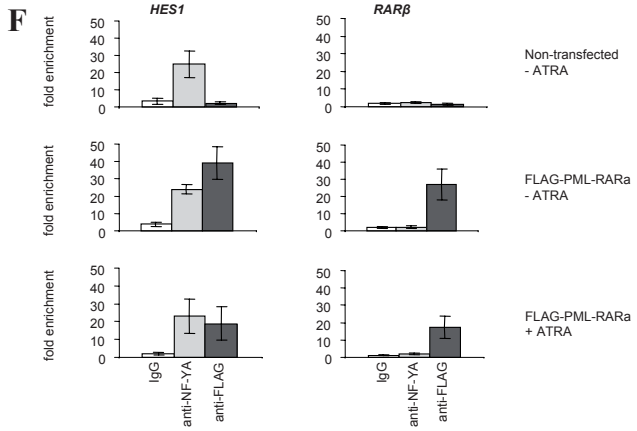


Figure 8. PML-RARα transactivates the *HES1* promoter without direct DNA binding. (A) Total RNA samples derived from non-stimulated and ATRA-stimulated NB4 cells (10-6 M ATRA was added for indicated time intervals) were subjected to RT-PCR using specific primers for the detection of HES1 mRNA. **(B)** Total cell lysates (75 mg) from non-stimulated and ATRA-stimulated NB4 cells (10-6 M ATRA was added for indicated time intervals) were subjected to immunoblot analysis using HES1 antibody. The same blot was reprobed with a β-actin antibody to demonstrate equal loading. The molecular weight (kDa) marker is indicated at the left. **(C,D,E)** Hep3B cells were transfected with a *HES1* promoter-luciferase reporter construct (pGL3-*HES1*), a control vector expressing Renilla-luciferase and vectors coding for the various proteins indicated in the figure. Background luminescence of the cells transfected with only the reporter construct in the absence of ATRA was set to 1. Transactivation was corrected for transfection efficiency measured by Renilla activity. Cells were cultured with (black bars) or without (white bars) ATRA. Mean values and standard deviations from three independent experiments are shown. **(F)** ChIP assays were performed both for the *HES1* promoter and for the *RARβ* promoter. Sonicated chromatin of HEK 293 cells transfected with FLAG-PML-RARα or of non-transfected HEK 293 cells were used for ChIP analysis using antibodies against NF-YA or FLAG or non-specific IgG as a control (n=4). Where indicated, cells were cultured in the presence of ATRA. Quantitative PCR was used to measure the amount of immunoprecipitated DNA of the *HES1* and *RARβ* promoter, which was compared to the control genomic sequences of *ALBUMIN*. The recovery of *HES1* and *RARβ* was plotted as the fold enrichment over the recovery of *ALBUMIN*.



proteins was bound to the *ID1* promoter in NB4 cells. To investigate this further we used a FLAG-tagged PML-RARα expression construct, allowing us to unequivocally identify binding by the PML-RARα fusion protein. HEK-293 cells were transfected with FLAG-PML-RARα (Figure 7A). As a negative control, ChIP was performed using non-specific IgGs. We compared the presence of (FLAG-tagged) PML-RARα and NF-Y on the two classical ATRA target genes *RAR*β and *p21*, and on *ID1* and *ID2*. Enrichment of all 4 promoters was found using anti-FLAG antibody, indicating that PML-RARα was bound to these four genes in intact cells (Figure 7A, compare upper and middle rows). Using anti-NF-Y antibody, enrichment of the *ID1* and *ID2* promoters was found but no enrichment of *RAR*β and *p21*, indicating that NF-Y was present on *ID1* and *ID2*, but not on the two classical ATRA target genes *RAR*β and *p21*. To see whether the presence of PML-RARα and NF-Y on the different promoters would be altered by the presence of retinoic acid, ChIP assays were performed in the presence and the absence of ATRA (Figure 7, middle and bottom rows). ATRA did not influence the binding of NF-Y or PML-RARα to these promoters showing that their binding is ligand-independent.

HES1 is transactivated by PML-RARα in a similar manner as *ID1* and *ID2*

Besides the ID proteins, Hairy-Enhancer of Split (HES) proteins (HES1-7) also act as transcriptional repressors of bHLH-regulated genes. Like ID proteins, HES proteins act, amongst other mechanisms, by sequestration of bHLH transcription factors into non-functional heterodimeric complexes [46, 47]. Analysis of the HES1 promoter sequence revealed a perfect CCAAT- and GC-box within 100bp upstream of the TATA-box. To investigate whether, besides the ID proteins, other inhibitors of bHLH transcription factors are regulated in APL cells, we assessed the expression level of HES1 during ATRA-induced granulocytic differentiation of APL cells. Total RNA and protein was isolated from the PML-RARα positive APL cell line NB4, cultured in the absence or presence of ATRA for several time intervals, and HES1 expression levels were measured by RT-PCR (Figure 8A) and Western blotting (Figure 8B). HES1 mRNA and protein expression levels were induced within half an hour of ATRA stimulation and reached maximum levels at 1 hour of ATRA stimulation. Subsequently, from 1 to 24 hours following ATRA stimulation, HES1 mRNA expression levels decreased about 100-fold and remained at this level during further differentiation. HES1 protein levels diminished to undetectable levels at 4 hours of ATRA stimulation. To investigated the mechanism that resulted in the transcriptional activation of *HES1* by PML-RARα. We cloned a 283 bp fragment of the *HES1* promoter, including the putative TATA-box, into a luciferase reporter construct. When expressed alone, RARα, RXR and PML were not able to transactivate this *HES1* promoter construct both in the absence and presence of ATRA. The combination RARα/RXR also did not transactivate the promoter. In contrast, PML-RARα transactivated the promoter more than 6-fold in an ATRA-dependent fashion (Figure 8c). Similar to the *ID1* and *ID2* promoters, PML-RARαΔR did, and PML-RARαΔCC and PLZF-RARα did not transactivate the *HES1* promoter (Figure 8d). Transactivation of *HES1* by PML-RARα was strongly decreased in the presence of dominant-negative NF-YA (Figure 8E). This indicates that PML-RARα-dependent transactivation of the *HES1* promoter is also dependent on a functional NF-Y complex. ChIP assays using chromatin from PML-RARα transfected cells revealed a clear increase in *HES1* promoter recovery when compared to untransfected cells, indicating that PML-RARα is present on the *HES1* promoter (Figure 8F). The ChIP assays also revealed the presence of NF-Y on the

HES1 promoter. We conclude that the *HES1* promoter is regulated in a similar manner as the *ID1* and *ID2* promoters.

Together, these data show that there are two different mechanisms by which PML-RARα may regulate the transcription of target genes (Figure 9). The first class of target genes consists of genes that contain a retinoic acid response element in their promoter. These genes are normally regulated by unrearranged retinoic acid receptors and may be deregulated by PML-RARα in a dominant-negative manner in the absence of ligand. In the presence of high concentrations of ligand, their expression is restored. The second class of target genes consists of genes that are not normally regulated by retinoic acid receptors. Fusion of the RARα moiety to PML renders these genes responsive to retinoic acid, which defines a novel gain-of-function for the PML-RARα fusion protein.

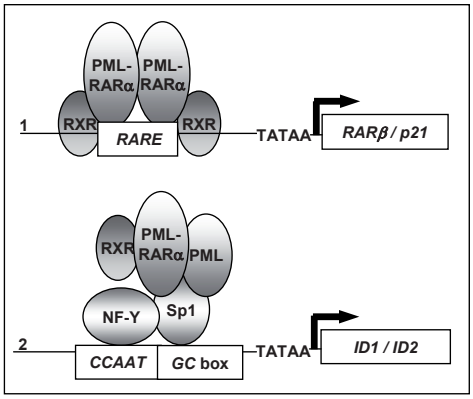


Figure 9. Dominant negative and gain-of-function model for PML-RARα. (1) Genes that are regulated through a retinoic acid responsive element (RARE) may be bound by PML-RARα. Competition with normal, unrearranged retinoid receptors results in a dominant-negative silencing of the gene by PML-RARα in the absence of ligand. Addition of high dose retinoic acid may reverse the silencing, allowing transcription. **(2)** Sp1 and NFY regulated genes may be targeted by PML-RARα through interaction with Sp1. Tethering of PML-RARα to these promoters renders them responsive to retinoic acid, representing a gain-of-function for the PML-RARα fusion protein.

Discussion

The successful treatment of APL with high dose retinoic acid has shown that the differentiation block of the malignant cells can be overcome, leading to terminal differentiation of the leukemic cells and disappearance of the disease. As this is one of the first examples of successful differentiation-induction therapy in cancer, many studies focused on the molecular mechanisms that contribute to the transformation to leukemia, and on the mechanisms that mediate the retinoic acid-induced differentiation of the cells. PML-RARα was shown to interfere with the expression of normal RARα target genes. RARα is an important modulator of granulopoiesis and acts either by direct binding to the DNA, or through interaction with other transcription factors. In this report we show that PML-RARα interacts with Sp1 and may interfere with the expression of genes that are not normally regulated by retinoic acid receptors. Previously, it was shown that both unrearranged PML [48] and unrearranged RAR/RXR complexes [22, 23] are able to interact with Sp1. Therefore, the interaction of PML-RARα with Sp1 could be mediated by the RARα part as well as the PML part of the fusion protein. Here we show that PML-RARα binds Sp1 and that this binding is less efficient when the coiled-coil domain is deleted. The lack of response of the *ID1* and the *ID2* promoters to RARα/RXR, PLZF-RARα and PML-RAR/ΔCC (Figures 2 and 5) suggests that the PML part of the fusion protein is essential for regulating transcription.

We show that PML-RARα physically interacts with Sp1 in the absence of DNA

(Figure 6). Roder et al. have shown that NF-Y and Sp1 interact physically in the absence of DNA. This suggests that the PML-RARα/Sp1/NF-Y complex may form before binding to DNA. Figure 3C suggests that the PML-RARα/Sp1/NF-Y complex still binds, although less efficiently, to the ID1 promoter when one transcription factor binding site is mutated. Figure 4C shows that the presence of a dominant negative form of NF-Y abolishes transactivation of the *ID1* promoter by PML-RARα. We hypothezise that this dominant negative form of NF-Y disrupts the PML-RARα/Sp1/NF-Y complex and thereby impairs binding to and transativation of the *ID1* promoter. We propose a model in which PML-RARα binds to a DNA-bound Sp1-NF-Y complex, rendering the expression of these genes sensitive to ATRA, and defining a novel gain-of-function for the fusion protein (Figure 7B). In the ChIP experiments, we show that unrearranged PML is recruited to the *ID1* gene (Figure 6C). The physiological meaning of this remains unclear as no effect of PML was observed in the transactivation assays.

As Sp1 is involved in the transcriptional control of various myeloid-specific genes [49, 50] deregulation of its target genes may be relevant in APL. For *ID1* and *ID2*, a role in myelopoiesis and in APL was described previously. Overexpression of ID1 or ID2 in NB4 cells inhibits their proliferation and induces a G0/G1 arrest [31]. In addition, ectopic expression of ID1 in CD34+ cells inhibited eosinophil development, whereas neutrophilic differentiation was enhanced. Expression of ID2 accelerated the definitive maturation of myeloid cells [51]. Other genes may be targeted by a similar mechanism as well. Overexpression of both ID1, ID2 and HES1 may reveal whether simultaneous inhibition of a wider range of HLH factors may have an even more profound effect on differentiation.

The promoter of the important retinoic acid responsive gene C/EBPβ was transactivated by PML-RARα, but also lacks a consensus RARE within the tested region [52, 53]. Possibly, also this gene is (de)regulated by tethering of PML-RARα to the promoter through protein-protein interactions rather than by direct DNA-binding.

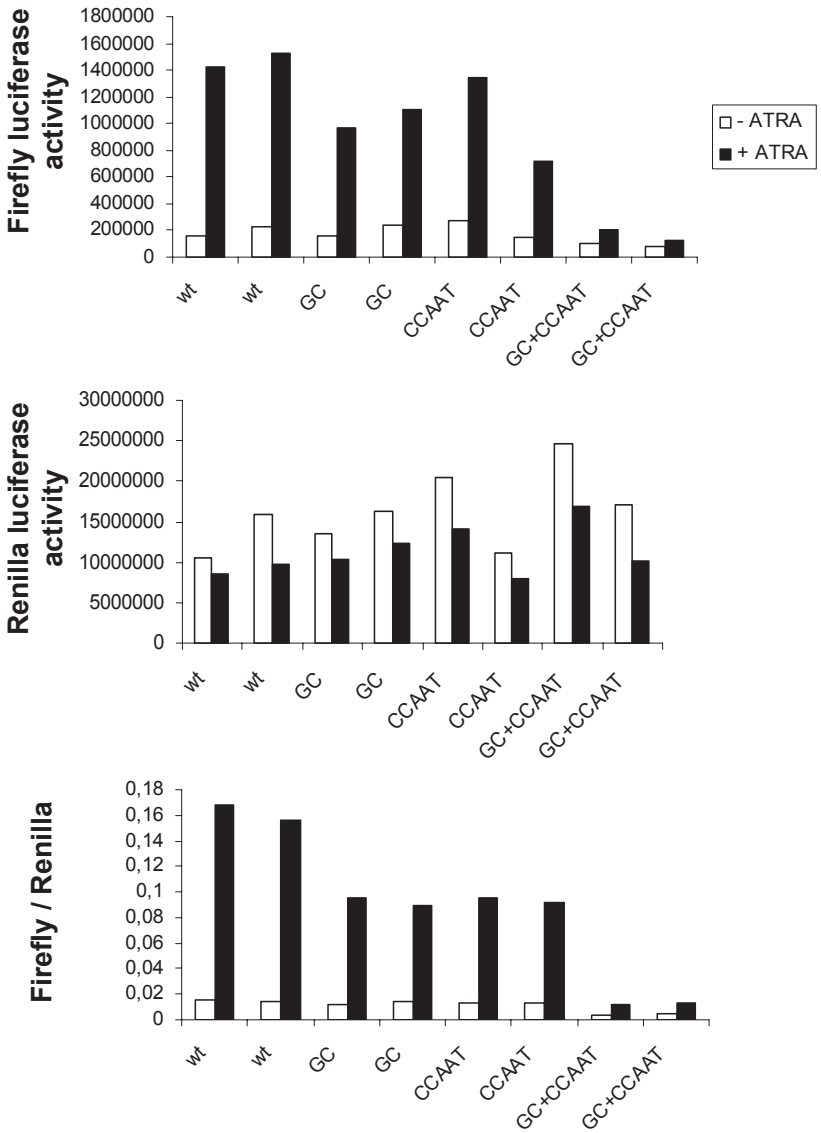
Apart from the DNA-binding domain from RARα, the coiled-coil domain of PML was shown to be important for optimal induction of terminal differentiation by the fusion protein [54]. This suggested that homodimerization of PML-RARα is an important feature of the fusion protein, but might also indicate that other protein-protein interactions mediated through the PML part are involved. Several alternative RARα fusion proteins occur at low frequency (2% of the cases) in APL patients, in which the same part of RARα is fused to other proteins than PML (reviewed in [44]). Common to the various RARα partner proteins is the presence of a dimerization domain, suggesting that homodimerization is an important property of these fusion proteins. However, depending on the fusion partner, the sensitivity to retinoic acid differs suggesting a broader role for the RARα partner protein than just the provision of a dimerization domain. Specifically, PLZF-RARα positive leukemia appears to be more resistant to ATRA, which has been explained by the recruitment of corepressors by the PLZF-part of the fusion protein that are not released upon treatment with ATRA [15, 55]. This insensitivity could be reversed, as treatment with a combination of ATRA and G-CSF induced granulocytic differentiation in a synergistic manner [56]. Also in transgenic mice, different types of disease with different sensitivities to ATRA developed for the various fusion proteins [7, 30]. Furthermore, when RARα fusion proteins were made by the coupling of RARα to artificial dimerization domains, several characteristics of PML-RARα were recapitulated in vitro, but in vivo these proteins induced leukemia with very low efficiency [57]. Interestingly, when these

fusion proteins were expressed in a *PML* -/- background, the animals did still not develop leukemia efficiently, leading to the conclusion that the mere combination of disruption of PML and dimerization of RARα does not recapitulate the full oncogenic potential of PML-RARα, and that the fusion protein has gain-of-function characteristics. Together, this showed that dimerization of RARα is important but not sufficient to reproduce the complete phenotype of PML-RARα.

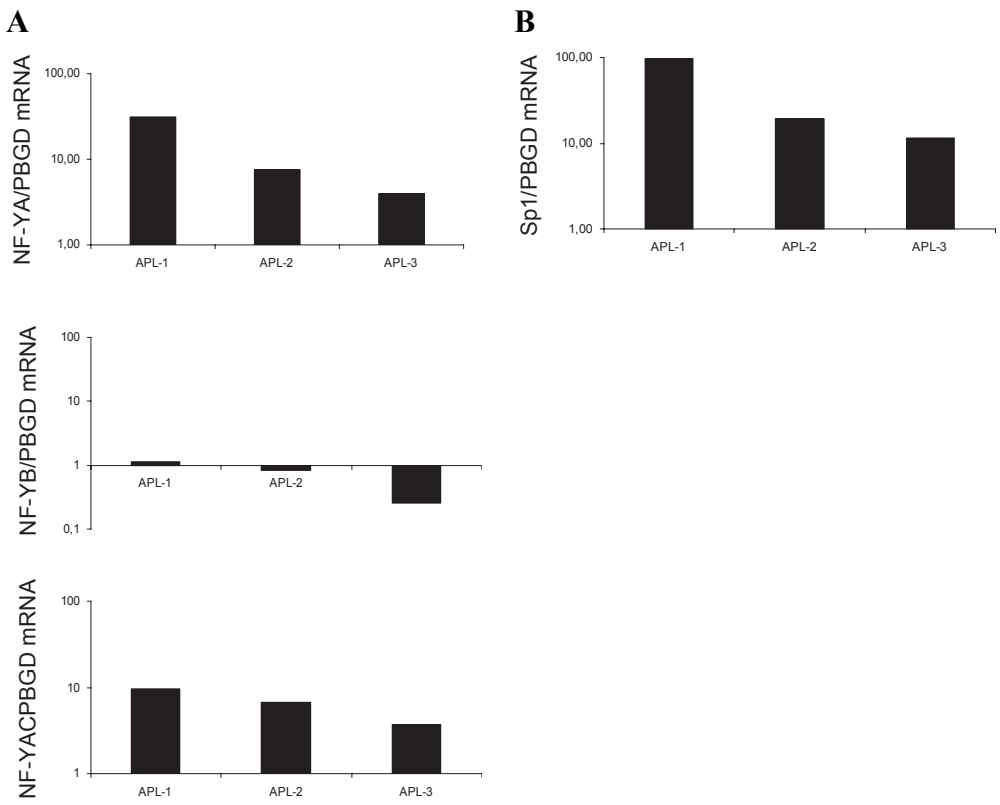
It remains to be investigated whether PML-RARα recruits co-repressors to the promoters of Sp1 and NF-Y target genes. This would lead to repression of gene expression in the absence of ATRA, possibly contributing to transformation of the cells, similar to the effect of PML-RARα on regular retinoic acid receptor target genes. So far, we did not observe important downregulation of ID1 and ID2 mRNA in freshly isolated leukemia cells from APL patient cells compared to non-APL acute myeloid leukemia cells, suggesting that this is not the case (data not shown). However, as ID1 and ID2 expression in most leukemic samples was low, further studies are required.

Additionally, we do not exclude the presence of RXR in the PML-RARα/Sp1/NF-Y complex. RXR is an essential part of the PML-RARα complex during transformation and synergy has been shown between ATRA and RXR specific agonist on transctiptional activation (Zhu et al 2007, Perez et al. Kamashev et al Jansen et al.). Therefore, RXR specific agonist may also effect transcription of Sp1 and NF-Y target genes.

In summary, we define a novel, Sp1 and NF-Y-dependent mechanism by which the PML-RARα fusion protein interferes with gene transcription. This implicates that PML-RARα (de)regulates an additional class of genes that are normally not regulated by retinoid receptors and defines a gain-of-function for the PML-RARα fusion protein.



Supplemental figure 1. Transactivation data before correction. Raw luciferase activaties are shown for one transactivation experiment from figure 3C. The *ID1* promoter, *wt* or with a mutated GCbox and/or CCAATbox, was cloned in front of the *firefly* gene. A *renilla* Expression construct was co-transfected for correction. Note that the correction does not qualitivily change the results.



Supplemental figure 2. NF-Y and Sp1 are expressed in APL cells. MRNA expression levels of NF-YA **(A)** and Sp1 **(B)** were determined in mononuclear cells from 3 APL patients. Bone marrow samples were obtained from t(15;17) APL patients following informed consent. Mononuclear cells were isolated by density gradient centrifugation using ficoll (1.077 g/ml, Sigma). Cells were taken up in RNA-bee (ISO-TEX Diagnostics, Friendswood, USA). RNA was used as template in a RT-DNA reaction as described before (de vries br j cancer 1999). Quantitative PCR was performed with the ABI/PRISM 7700 Sequence Detection system (ABI/PE, Foster City, Ca, USA). As a reference gene we used PBGD (de vries br j cancer 1999). The primer/probe sets for detecting NF-YA and Sp1 were from Applied biosystems (Foster City, Ca, USA). PCRs were performed in universal master mix (Roche).

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The PML-RARα fusion protein enhances MDM2 protein levels

Abstract

APL is characterized by the PML-RARα fusion protein. We investigated the effect of PML-RARα on MDM2 expression. Ectopic expression of PML-RARα increased MDM2 protein levels. Degradation of PML-RARα correlated with downmodulation of MDM2. As PML-RARα may inhibit p53 function by enhancing MDM2 levels we tested whether MDM2 inhibition would trigger apoptosis. Treatment of leukemia cells with the MDM2 antagonist Nutlin-3 resulted in induction of apoptosis. We conclude that PML-RARα may contribute to leukemogenesis by enhancing MDM2 protein levels which results in inactivation of p53. Targeting MDM2-mediated inactivation of p53 could be a potential option to treat retinoic acid resistant APL.

Introduction

Disruption of p53 function is a common trait of malignant cells, and *TP53* is presently considered to be the most frequently mutated gene in human cancer [1]. In leukemia, mutations in the *TP53* gene are less frequent, occurring in approximately 10% of the cases [2]. Therefore, it has been suggested that in hematological malignancies the function of p53 is deregulated by other mechanisms. One of the most important negative regulators of p53 is MDM2. MDM2 binds to p53, blocks p53-mediated transactivation and targets p53 for proteasomal degradation [1]. In leukemia, *mdm2* gene amplification was not found, but overexpression of MDM2 mRNA may occur in up to 50% of the cases [3]. Apart from deregulated mRNA expression, altered MDM2 function may occur in leukemia by other means.

Another regulator of p53 function is $p14^{arf}$. $p14^{arf}$ induces the p53 pathway by inhibiting the activity of MDM2. We have shown that the transcription of $p14^{arf}$ is directly repressed by the AML1-ETO fusion gene [4], suggesting a direct link between the t(8;21) specific oncogene and deregulation of the p53 pathway.

Here we study MDM2 in acute promyelocytic leukemia (APL). APL is characterized by the PML-RARα fusion protein which usually results from a reciprocal t(15;17) chromosome translocation [5]. Earlier, it was shown that the wild type PML protein regulates the p53 pathway at several levels. Wild type PML localizes to the multiprotein nuclear substructures termed nuclear bodies. P53 protein is recruited to these nuclear bodies by PML upon oncogenic stress, where p53 is activated by acetylation by CBP/p300. In addition, binding of PML to p53 antagonizes the binding of MDM2, thereby stabilizing the p53 protein. Finally, PML is able to sequester MDM2 to the nucleolus, resulting in diminished p53 degradation [6]. PML-RARα does not bind p53 because of loss of the carboxy-terminus of PML in the fusion protein. PML-RARα expression does however impair p53 activation and triggers p53 degradation. This process is dependent on several factors. Wild type PML protein needs to be present as a bridging factor between PML-RARα and p53. PML-RARα needs to recruit HDAC activity which is dependent on a domain in the RARα part of the fusion protein. Finally, proteasomal degradation is dependent on the presence of MDM2 [7]. Expression of the PML-RARα fusion protein causes deacetylation of p53, resulting in repression of transcriptional

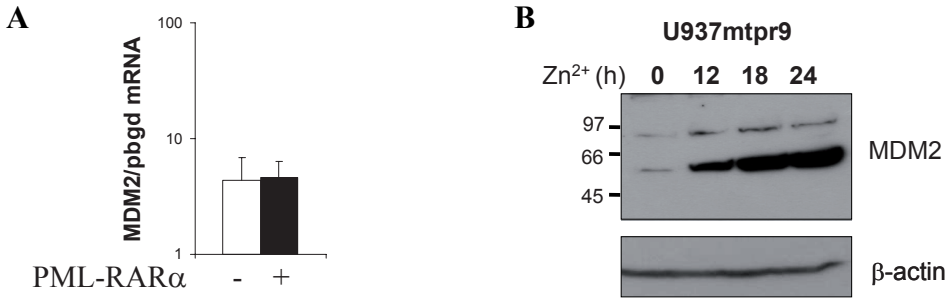

Figure 1. PML-RARα induces MDM2 protein levels and not mRNA levels. (A) U937-pr9 cells were treated with Zn2+ to induce PML-RARα expression. Cells were harvested 0 and 24 hours after Zn2+ treatment. Using quantitative PCR MDM2 mRNA levels, relative to PBGD mRNA levels, were determined. ATRA was used at 10^{-6} M, $ZnSO_4$ at 100 μM. **(B)** U937-pr9 cells were treated with Zn2+ to induce PML-RARα expression. Cells were harvested after the indicated period of time in SDS-loading buffer. Protein was resolved on SDS-PAGE. Immunostaining was done with MDM2 and β-actin specific antibody.

activity, and MDM2-dependent degradation of p53 [7]. However, the effect of PML-RARα on MDM2 is unknown.

Materials and Methods

Reagents
The selective MDM2 antagonist, Nutlin-3 (racemic, EMD Biosciences, San Diego, CA), was dissolved in ethanol (10 mM) and was stored at -20ºC. ATRA (Sigma, St Louis, MO) was used at 10^{-6} M, $ZnSO_4$ at 100 μM.

Cell lines and APL samples
NB4 cells and U937-pr9 cells (kindly provided by Dr. P.G. Pelicci) were cultured in RPMI 1640 medium (Gibco, Gaithersburg, MD) supplemented with 10% FCS (Gibco). Bone marrow samples were obtained from t(15;17) APL patients following informed consent. Mononuclear cells were isolated by density gradient centrifugation using ficoll (1.077 g/ml, Sigma). The percentage of APL cells was determined by staining for PML-RARα microspeckles [5] using anti-PML (PG-M3, Santa Cruz, CA) and was in all cases > 80%. Cells were cultured in RPMI 1640 medium supplemented with 20% FCS.

Quantitative PCR
RNA was isolated using RNAzol (Campro Scientific, Veenendal, the Netherlands) according to the manufacturer's instructions. For cDNA synthesis, 1 μg total RNA was reverse-transcribed using random hexanucleotide primers. Quantitative PCR was performed with the ABI/PRISM 7700 Sequence Detection system (ABI/PE, Foster City, Ca, USA) using Sybr Green PCR (Applied Biosystems). As a reference, expression of the *PBGD* was measured [8]. The primers for detecting $p14^{arf}$ were forward: 5'-AGCAGCCGCTTCCTAGAAGAC, reverse 5'-CACGGGTCGGGTGAGAGT. Primers for detecting MDM2 were: forward 5'-ACCACCTCACAGATTCCAGCTT, reverse 5'-GCACCAACAGACTTTAATAA CTTCAAA.

Cell proliferation and apoptosis analysis
Cells were washed with HBSS and incubated in 100 μL annexin V buffer (10mM HEPES, 140 mM NaCl and 2.5 mM $CaCl_2$, pH 7.4) with 5 μL annexin V (Molecular Probes,

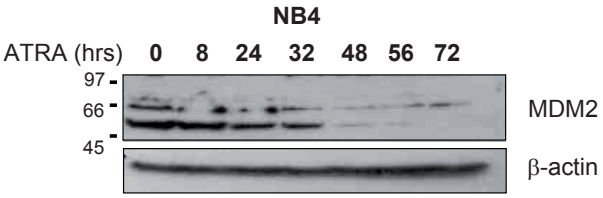

Figure 2. ATRA treatment of APL cell line NB4 results in downregulation of MDM2 protein. NB4 cells were treated with ATRA to induce terminal differentiation and degradation of PML-RARα. Cells were harvested after the indicated period of time in SDS-loading buffer. Protein was resolved on SDS-PAGE. Immunostaining was done with MDM2 and β-actin specific antibody.

Leiden, the Netherlands). After 30 min. another 400 μL of annexin V buffer and propidium iodide (PI, 25 μg/ml) was added. Samples were analysed on a flow cytometer (Beckman coulter).

Western blot analysis.
For Western blot, cells were FACS-sorted and directly taken up in sample buffer (0.125 M Tris-HCL (pH 6,8), 1% SDS, 5% 2-mercaptoethanol, 5% glycerol). Extracts were separated on a 10% SDS-polyacrylamide gel, and transferred to a PVDF membrane. MDM2 was visualised using specific antibody (SMP14, Santa Cruz, CA).

Results and discussion
To study the effect of the PML-RARα fusion protein on MDM2 expression we used the U937-pr9 cell line that harbors a Zinc-inducible PML-RARα expression cassette [9]. First, we confirmed the induction of PML-RARα in these cells by zinc. The PML protein is normally localised to large nuclear bodies and is delocalised, in the presence of the PML-RARα fusion protein, to multiple small dots, termed microspeckles [5]. Twelve hours after addition of zinc to the culture medium, the appearance of microspeckles was seen in the cells (data not shown). Using quantitative PCR, we analysed the amount of MDM2 mRNA , relative to the control gene PBGD. No up- or downregulation of MDM2 mRNA levels was found upon the expression of PML-RARα (Figure 1A). In contrast, the induction of PML-RARα resulted in a clear upregulation of the p60 form of MDM2 protein (Figure 1B). The p60 protein is an isoform of MDM2 that is produced by caspase cleavage of the full length MDM2 protein [10]. When APL cells are treated with all-trans retinoic acid (ATRA), the cells differentiate towards mature granulocytes, which is accompanied by degradation of the PML-RARα fusion protein [11]. Therefore, we studied the level of MDM2 protein expression in NB4 cells before and after stimulation with ATRA. The p60 form of MDM2 was clearly detectable in untreated NB4 cells (Figure 2). Treatment with ATRA resulted in a clear downregulation of MDM2 p60 protein levels (Figure 2). As PML-RARα expression had no effect on MDM2 mRNA levels, this suggests that PML-RARα expression results in increased MDM2 p60 protein levels through a transcription independent process. High expression levels of the p60 form of MDM2 have been shown to inhibit p53 transcriptional activity [12]. The p60 form of MDM2 has lost its carboxy-terminal domain, which contains the RING domain necessary for ubiquitination of p53. However the p60 form of MDM2 retains its domains to bind p300 and p53. So, p53 acetylation in APL cells may be impaired both by HDAC recruitment by PML-RARα and high levels of MDM2 p60 protein. The p60 form of MDM2 has been found in several tumor cell lines, and in breast tumors [10]. Therefore, tumor cells overexpressing the p60 form of MDM2 may be protected from p53-mediated apoptosis.
To study whether upregulation of MDM2

by PML-RARα is functionally important in APL cells, we used Nutlin-3. Nutlins are a novel class of synthetic small molecules, which disrupt the binding of MDM2 to p53, thereby inhibiting p53 degradation and activating p53-dependent apoptotic pathways [13]. The relevance of this finding was recently underscored by the fact that restoring endogenous p53 expression in murine lymphomas, sarcomas and liver carcinomas resulted in tumor regression while not affecting normal tissues. This indicates that these tumors rely on sustained p53 inactivation for their growth and that reactivation of p53 function is a potential avenue for therapeutic development [14].

In both the U937 and NB4 cell lines, p53 is not functional [7]. Therefore, we tested the effect of Nutlin-3 on freshly isolated leukemia cells from APL patients (>80% pure). Apoptosis was determined by staining for Annexin V and PI. After one day of culture, cells were exposed to Nutlin-3 or vehicle control. When cells were treated with 0, 1, 5 and 12.5 μM of Nutlin-3, a time- and dose-dependent increase in apoptosis was observed (Figure 3A). Subsequently, we tested leukemic cells from five different APL patients using 5 μM of Nutlin-3 (Figure 3B). In four patients, a clear increase in apoptosis was observed within 48 hours. In one patient, no significant effect of Nutlin-3 was seen. The APL cell line NB4, which contains a mutated p53 gene, was insensitive to Nutlin-3. This data indicates that inhibition of MDM2 in APL cells may activate the p53-apoptotic pathway. Earlier, Kojima *et al.* [15] showed that Nutlin-3 induces p53-dependent apoptosis in non-APL, acute myeloid leukemia (AML) cells that do not carry *TP53* mutations. In addition, they showed that the leukemic cells were more sensitive to this compound than normal $CD34^+$ colony forming cells. This indicates a broad applicability of Nutlins in the treatment of leukemia. The sensitivity to Nutlins suggests that in many AML patients, p53 remains functional, but that the p53 pathway is defective, upstream of p53.

How PML-RARα enhances MDM2 protein levels remains unclear. In PML-RARα positive cells PML localises into microspeckles instead of into normal PML nuclear bodies. We hypothesise that, since PML is able to bind MDM2 and sequester MDM2 to the nucleolus, MDM2 stabilization is due to a loss of function of PML in PML-RARα positive cells. In summary, we show that MDM2 proteins levels are upregulated by PML-RARα. As the functional antagonist of MDM2 (Nutlin-3) induces p53-dependent apoptosis in these cells, this suggests that enhancing MDM2 protein levels by PML-RARα is an important property of the fusion protein that may contribute to the malignant transformation of the cells.

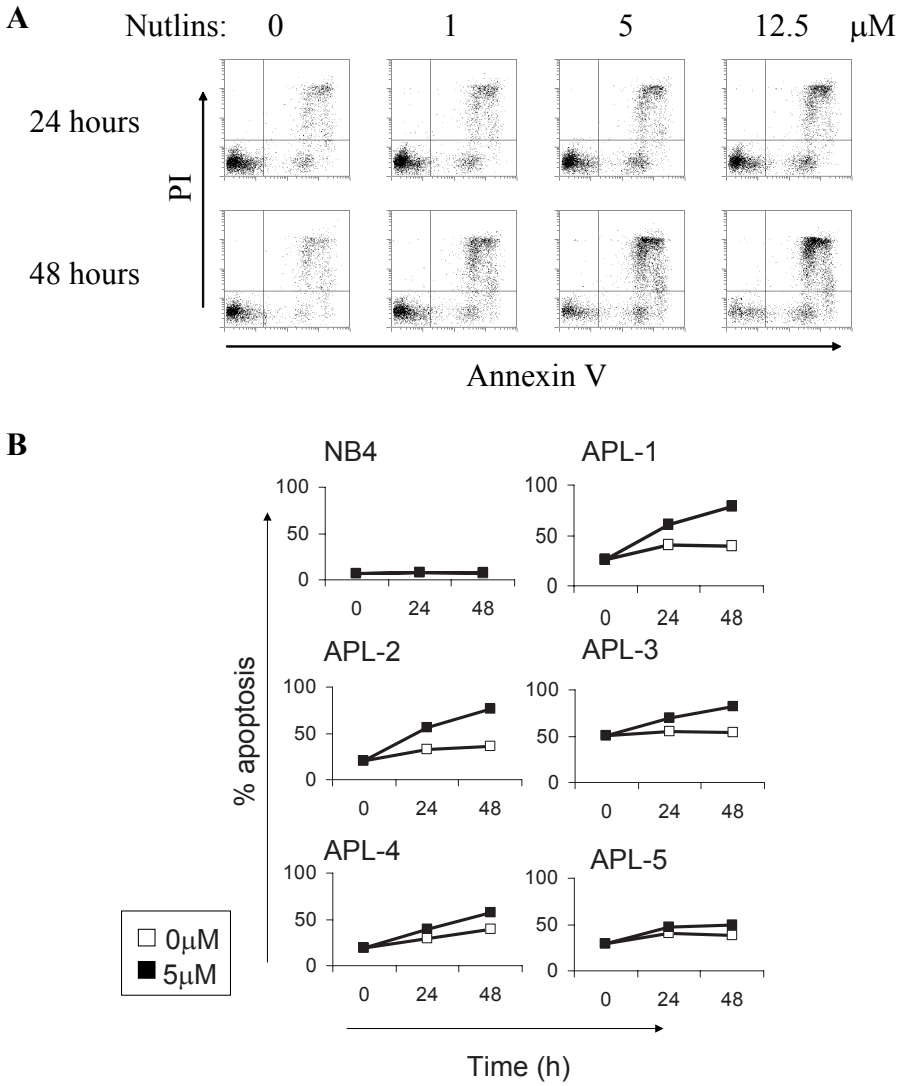

Figure 3. MDM2 antagonists induce apoptosis in APL patient cells. Primary APL cells from patients were treated with the MDM2 antagonist Nutlin-3 or vehicle control. Bone marrow samples were obtained from t(15;17) APL patients. Mononuclear cells were isolated by density gradient centrifugation using ficoll (1.077 g/ml, Sigma). The percentage of APL cells was determined by staining for PML-microspeckles and was in all cases > 80%. Cells were cultured in RPMI 1640 medium supplemented with 20% FCS for the indicated period. Nutlin-3 or vehicle control was added to the medium. Cells were harvested and washed with HBSS and incubated with annexin V and propidium iodide. Samples were analysed on a flow cytometer **(A)** APL cells from one patient were treated with different concentrations of Nutlin-3. **(B)** Primary APL cells from 5 different patients and NB4 cells were treated with the MDM2 antagonist Nutlin-3 or vehicle control. After 0, 24 and 48 hours cells were analysed by flow cyometry.

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The transcription factor NF-Y regulates the proliferation of myeloid progenitor cells

Abstract

The transcription factor NF-Y regulates the expression of several hematopoietic genes. To determine the biological role of NF-Y in hematopoiesis we expressed a dominant negative form of NF-Y in murine bone marrow cells by retroviral transduction. Inhibition of NF-Y function resulted in a significant reduction of granulocytic (CFU-G) and monocytic (CFU-M) colony formation by 70% and 44 %, respectively. To study this reduction in more detail, we analysed the effect of dominant negative NF-Y on proliferation, differentiation and apoptosis. In more mature, MAC-1 positive cells, no differences in proliferation, differentiation and apoptosis were observed between dominant negative NF-Y- and vector-transduced cells. In contrast, the expansion of MAC-1 negative cells was completely abrogated by the expression of dominant negative NF-Y. Moreover, the inhibition of NF-Y function resulted in a significant increase in apoptosis in MAC-1 negative cells. Competitive transplantation of BM cells to irradiated mice, showed a repopulation advantage of vector-transduced cells versus dominant negative NF-Y transduced cells. Our data show that NF-Y regulates the proliferation of myeloid progenitor cells.

Introduction

During hematopoiesis, different maturation stages are defined by the expression of different sets of genes, a process regulated by specific transcription factors. Transcription factors that are essential for the outgrowth of pluripotent stem cells have been identified, such as AML-1 and SCL [1]. Other transcription factors were shown to be essential for more lineage restricted progenitors cells. For example, C/EBPα for granulocytic cells [2]. Mutations in various of these transcription factors are recurrently found in leukemia [1].

Recent studies have shown that the transcription factor, Nuclear Factor Y (NF-Y), regulates the expression of several hematopoietic (stem) cell genes. NF-Y regulates CD34 [3], the human telomerase RNA gene [4] and HOXB4 expression [5]. HOXB4 expression increases stem cell expansion [6]. Other known targets of NF-Y are cell cycle related genes, like Cyclin B1 which is involved in the G_2/M transition. Using chromatin immunoprecipitation, NF-Y was shown to bind to the *cyclin B1* promoter [7]. Upon p53-dependent G_2-arrest, NF-Y mediates the inhibition of *Cyclin B1* and *B2* promoters [8-10].

NF-Y was identified as a protein that binds a conserved element in the MHC class 2 promoter called the Y box [1;11]. NF-Y binds to a CCAAT box enhancer element, consisting of the consensus G/A, G/A, CCAAT, C/G, A/G, G/C sequence [12]. NF-Y consists of three subunits NF-YA, -B and -C, and this trimeric complex is able to bind to the DNA and to the basal transcription machinery through binding of the TATA box-binding protein, TBP [13-15].

Deletion of both NF-YA alleles in mice, results in an incomplete and inactive NF-Y complex, causing embryonic lethality. Inactivation of NF-YA in mouse embryonic fibroblasts results in a block in proliferation, which is followed by apoptosis [16]. A few studies have been performed addressing the role of NF-Y in hematopoiesis. Mouse bone marrow (BM) cells overexpressing NF-YA are biased towards primitive hematopoiesis and show increased repopulating ability after bone marrow transplantation [17]. During macrophage differentiation NF-Y shows increasing binding activity to several promoters [18] and NF-Y has

been shown to mediate the IL-6 response in BM cells, which results in induction of macrophage differentiation [19]. We used a dominant negative form of NF-Y (YAm29) [20] to more precisely define the role of NF-Y in myelopoiesis.

Materials and Methods

DNA constructs
The dominant negative form of NF-YA (YAm29) in pSG5 was a generous gift from Dr. Mantovani [20]. pSG5-YAm29 was used as a template in a PCR reaction. Primers specific for NF-YA contained EcoR1 and Xho1 restriction sides. YAm29 was cloned in the retroviral vector pLZRS [21;22] using EcoR1-Xho1, leading to the following sequence of viral DNA: LTR-YAm29-IRES-GFP-LTR.

Transduction of murine bone marrow cells
Chemicals and cytokines were from Sigma (Zwijndrecht, the Netherlands) unless stated otherwise. Bone marrow was harvested from 6 weeks old, female CBA/CA mice. Cells were cultured for 2 days in pre-stimulation medium: IMDM medium (Invitrogen, Life Technologies, Breda, the Netherlands) supplemented with 10% FCS (Integro, Zaandam, the Netherlands), 1% penicillin and streptomycin solution; respectively 5000 units and 5mg/ml (Invitrogen), 7µl/l β-Mercapto-ethanol, 1% BSA, 1% nucleoside mix (0.1mg/ml cytidine, adenosine, uridine, guanosine, 2'deoxycytidin, 2'deoxyadenosine, thymidine, 2'deoxyguanosine), 10µg/mL bovine insulin (Invitrogen), 15µM chlorestorol, 15µM linolic acid, 0.62mg/mL human transferrin and the following recombinant cytokines: murine SCF 50ng/mL, murine IL-12 10ng/mL, murine IL-3 10ng/mL, human Flt-3 50ng/mL, human TPO 10ng/mL (ITK Diagnostics, Uithoorn, the Netherlands), α-TGF-β 1µg/mL (ITK Diagnostics).
Cells were plated onto retronectin-coated culture dishes (Takara, Cambrex Bio science, Heerhugowaard, the Netherlands,) and exposed to the supernatant of packaging, ecotropic Phoenix cells, transfected with the indicated vectors. Supernatant was mixed (1/1) with 2*pre-stimulation medium.

Clonogenic and liquid culture assays
24 hours after transfection BM cells were sorted for GFP positivity by FACS (Epics Elite, Beckman Coulter, Mijdrecht, the Netherlands). For the semi-solid culture 5000 cells were plated per mL methylcellulose medium: 1.2% methylcellulose in IMDM medium supplemented with 0.1mL/mL Horse Serum, 0.1mL/mL Pokeweed Mitogen Stimulated Mouse Spleen Conditioned Medium (PWM, Invitrogen), 50ng/mL SCF, 7µl/L β-mercapto-ethanol. Granulocyte colony-forming units (CFU-G) and monocyte colony-forming units (CFU-M) were counted 5 days later using an inverted microscope. For the liquid cultures BM cells were grown in the same medium as used for the colony assays, but lacking methylcellulose. Cells were grown in 96 well plates.

Transplantation of murine bone marrow cells
Transplantations were performed by injecting a 50/50 mixture of BM cells, transduced with YAm29 vector/empty vector, into irradiated mice (2 X 6 Gy at a 24 hours interval). For each transplantation, 0.25 X 10^6 sorted BM cells, transduced with YAm29, from C57BL6 mice ($CD45.2^+$) together with 0.25 X 10^6 sorted BM cells, transduced with empty vector, from C57BL6 mice ($CD45.1^+$) were used. Transplants were injected into the tail vein of recipients that were heterozygous for CD45.1 and CD45.2. Contribution from YAm29 and empty vector transduced cells were analysed 12 days after transplantation.

Differentiation analysis
For analysing the differentiation of the BM cells, cells were incubated with MAC-1 specific antibody (1:100, Pharmingen, the Netherlands, Alphen a/d Rijn). Just before

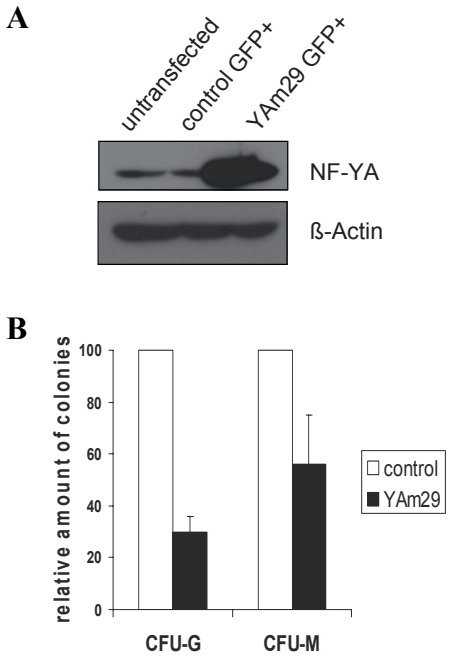


Figure 1. YAm29 inhibits clonogenic growth of mouse BM cells. (A) BM cells were transduced with empty vector (control) or YAm29 retrovirus, sorted for GFP expression and cell lysates were resolved on SDS-PAGE and stained using NF-YA specific antibody. After sorting there was clear expression of YAm29 in GFP positive cells. **(B)** BM cells were transduced with empty or YAm29 virus and GFP positive cells were plated in methylcellulose. 5 days later CFU-G and CFU-M colonies were counted. The amount of colonies is shown after transduction with YAm29 as a percentage relative to the amount of colonies after transduction with empty vector.

FACS analysis 50µL of flow-check beads (Beckman coulter) was added to each sample to count the cells.

Western blot analysis

For Western blot, cells were sorted and directly taken up in sample buffer (0.125 M Tris-HCL (pH 6,8), 1% SDS, 5% 2-mercaptoethanol, 5% glycerol). Extracts were seperated on a 12% SDS-polyacrylamide gel, and transferred to a PVDF membrane. NF-YA was visualised using specific antibody (C-18, Santa Cruz, Heerhugowaard, the Netherlands).

Results

Expression of dominant-negative NF-Y inhibits clonogenic growth of BM cells

A mutant form of NF-YA (YAm29), defective in DNA-binding was constructed by Mantovani et al.[20]. YAm29 was shown to have a dominant-negative effect on transcription regulation by wild type NF-Y. To determine the requirement of NF-Y in myelopoiesis, we first cloned the YAm29 cDNA in the LZRS vector [21;22]. This vector contains an YAm29-IRES-GFP expression cassette, allowing the identification of transduced cells. Primary mouse bone marrow (BM) cells were transduced with YAm29. As a control we transduced cells with an empty vector, containing only the GFP coding sequence. One day after transduction, GFP positive cells were sorted by FACS and analysed for YAm29 expression (Figure 1A). Clear protein expression of YAm29 was detected by Western blot analysis in the GFP positive fraction, in contrast to GFP negative cells or empty vector transduced cells. To determine the effect of YAm29 expression on colony formation, GFP+ cells were plated in methylcellulose and colonies were counted after five days. Transduction with YAm29 resulted in a reduction of granulocytic (CFU-G) and monocytic (CFU-M) colony formation by 70±6 % and 44±19% (n=3) respectively, compared to control cells transduced with empty vector alone (Figure 1B). In addition transduction with YAm29 resulted in a reduction of the size of both colony types (not shown). The data suggest that NF-Y is required for the proliferation of myeloid progenitor cells.

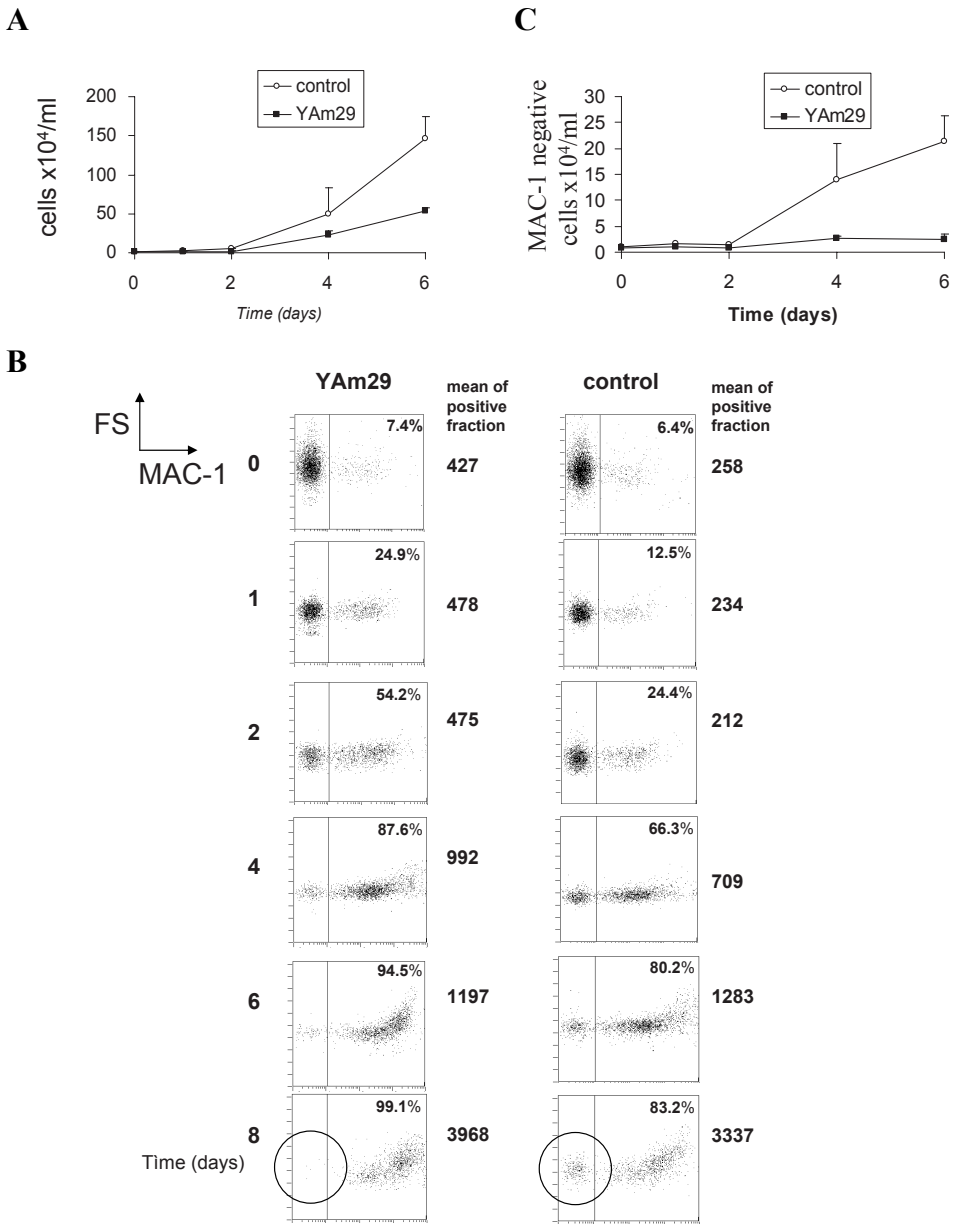


Figure 2. YAm29 inhibits proliferation of MAC-1 negative cells. (A) BM cells were transduced with control or YAm29 retrovirus (t= -1) and GFP positive cells were grown in liquid medium. Cells were counted on a flow cytometer by adding a constant amount of beads to each sample **(B)** transduced cells were incubated with MAC-1 antibody to discriminate between immature and mature myeloid cells. Shown are the FACS dot plots, forward scatter (FS) against MAC-1 expression, from day 1 to day 8. The X-mean is the mean intensity of the MAC-1 expression **(C)** The expansion of MAC-1 negative cells was calculated by multiplying the percentage of MAC-1 negative cells, from figure B, with the total amount of cells, from figure A.

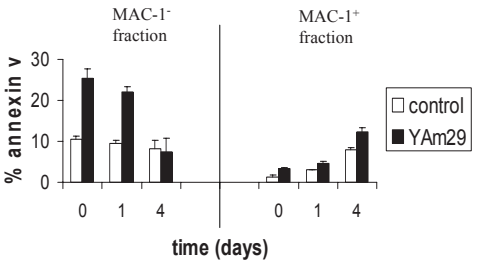


Figure 3. YAm29 increases apoptosis in MAC-1 negative cell fraction. BM cells were transduced with control or YAm29 retrovirus (t= -1) and GFP positive cells were grown in liquid medium. Cells were double stained with MAC-1 antibody and annexin V and analysed by FACS to assess the percentage of apoptotic cells.

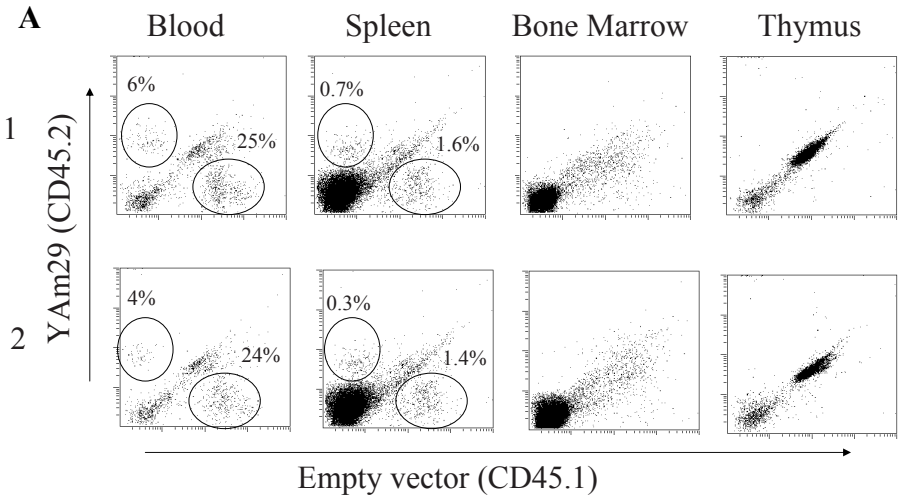
Expression of dominant-negative NF-Y inhibits the expansion of myeloid progenitor cells *in vitro*

To quantify the inhibitory effect of YAm29 on proliferation, FACS-sorted cells were grown in liquid culture. The empty vector transduced population expanded faster than the YAm29 transduced population, resulting in a three-fold difference on day 6 (Figure 2A). To investigate the effect of YAm29 on mature and immature cells, the differentiation marker MAC-1 was used. MAC-1 is expressed on maturing myeloid cells, beyond the colony-forming unit (CFU) stage [23;24]. The percentage of MAC-1 positive cells increased more rapidly within the YAm29 transduced population than in the control population. After 8 days, all YAm29 transduced cells were MAC-1 positive (Figure 2B), while in the control population around 17% of the cells were still MAC-1 negative. This suggested that the immature cells in the control population still proliferated and in the YAm29 transduced population proliferation was inhibited. The expansion of the MAC-1 negative cell fraction was calculated by multiplying the total amount of cells with the percentage of MAC-1 negative cells. This revealed that proliferation of the YAm29 transduced population was completely impaired, whereas the MAC-1 negative population in the empty vector transduced cells still expanded (Figure 2C). The intensity of the MAC-1 marker, determined by taken the X-mean (Figure 2B), which correlates with the maturation stage of the cells, increased at an almost comparable pace for both populations. This suggested that YAm29 expression did not severely affect differentiation.

To investigate whether the loss of expansion in YAm29 transduced BM cells was accompanied by increased apoptosis we stained the BM cells with annexin V. Within the MAC-1 negative population, 9-10% annexin V positive cells were found the first 2 days after transduction of the empty vector. 23-25% of annexin V positive cells were found the first 2 days after transduction with YAm29 (Figure 3). This higher percentage of annexin V positivity was not found in the YAm29 transduced, MAC-1 positive population. These data suggested that loss of NF-Y function resulted in increased apoptosis, specifically in myeloid progenitor cells.

Expression of dominant negative NF-Y inhibits expansion of progenitor cells *in vivo*

Expression of YAm29 resulted in increased apoptosis and loss of proliferation of myeloid progenitor cells *in vitro*. To investigate the requirement for NF-Y *in vivo*, we transplanted mice with YAm29 expressing BM cells using a competitive BM transplantation assay. Irradiated CD45.1/CD45.2 heterozygotic mice were transplanted with YAm29 transduced CD45.1 BM cells and empty vector transduced CD45.2 BM cells in a 50/50 ratio, allowing the discriminations between YAm29 transduced, empty vector transduced and recipient cells. Analysis of the mice on day 12 showed a predominance of the cells transduced with empty vector versus YAm29 transduced cells (respectively ±5% compared to ±28% in blood, Figure 4A). Both the CD45.1 and the CD45.2



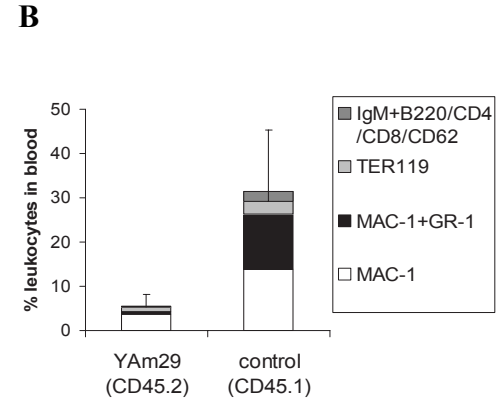
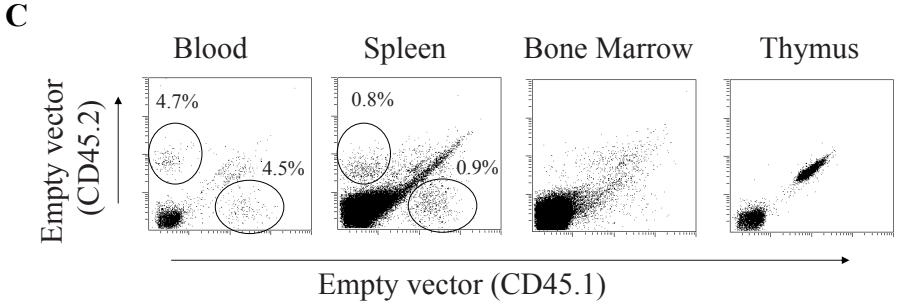


Figure 4. YAm29 inhibits the expansion of BM cells in a competitive transplantation assay. (A) CD45.2$^+$ BM cells were transduced with YAm29 retrovirus. CD45.1$^+$ BM cells were transduced with control retrovirus. GFP positive cells were transplanted, in 50/50 ratio, into irradiated (CD45.1$^+$ +CD45.2$^+$) recipients (n=3). Blood, spleen, bone marrow and thymus were isolated at day 12 and CD45.1$^+$ and CD45.2$^+$ and CD45.1$^+$ +CD45.2$^+$ leukocytes were counted. Shown are the FACS plots of 2 transplanted mice. **(B)** Leukocytes were stained with the indicated antibodies and analysed by FACS. Given are the mean values of three mice **(C)** Both CD45.2$^+$ BM cells and CD45.1$^+$ BM cells were transduced with control retrovirus. GFP positive cells were transplanted, in 50/50 ratio, into irradiated recipients (CD45.1$^+$ +CD45.2$^+$). Blood, spleen, bone marrow and thymus were isolated at day 12 and CD45.1$^+$ and CD45.2$^+$ and CD45.1$^+$ +CD45.2$^+$ leukocytes were analyzed by flow cytometry.



expressing leukocytes at day 12 were mainly of the myeloid lineage (±90% MAC-1 positive cells, figure 4B). As a control we transplanted mice with empty vector CD45.1 BM cells and empty vector transduced CD45.2 BM cells in a 50/50 ratio. This resulted in equal growth of both the CD45.1 and the CD45.2 BM cells (Figure 4C). Together these data indicate that suppression of NF-Y reduced the proliferation of myeloid progenitors.

Discussion

Several hematopoietic genes are a target of the transcription factor NF-Y, suggesting a role for NF-Y in hematopoiesis. Because *NF-YA* -/- mice are embryonic lethal [16], the use of a dominant negative form of NF-Y offers an elegant way to study the requirement for NF-Y in hematopoiesis. We transduced mouse BM cells with a dominant-negative form of NF-YA. YAm29 has been shown to prevent the formation of a functional NF-Y complex [20]. Blocking the function of NF-Y in BM cells, *in vitro,* resulted in a significant reduction of granulocytic (CFU-G) and monocytic (CFU-M) colony formation in size and number. To explain this loss of clonogenic potential, transduced BM cells were analysed in a liquid assay. BM cells transduced with YAm29 were still able to become MAC-1 positive, suggesting maturation was not severely affected. However, YAm29 transduction completely inhibited the expansion of the immature, MAC-1 negative population. This suggests that YAm29 inhibits expansion of the myeloid progenitor cells, explaining the overall decrease in myeloid cells in the liquid and colony assays upon YAm29 expression and indicating that functional NF-Y is needed for expansion of the myeloid progenitor cells. Using a competitive transplantation assay we studied the requirement for NF-Y for proliferation of BM cells *in vivo.* In these experiments, YAm29 also had a negative effect on the expansion of progenitor cells in comparison to empty vector transduced cells.

Although NF-Y regulates the expression of several genes involved in progression through the cell cycle and YAm29 expression blocks expansion of the MAC-1 negative cells, there is still an overall increase in the number of BM cells after transduction of YAm29. This indicates that YAm29 expression does not block cell cycle progression in all cells. Possibly the effect of YAm29 expression depends on the inhibitions of the expression of genes that are involved in maintaining a progenitor cell phenotype. One target gene of NF-Y is HOXB4. Expression of HOXB4 is found in immature hematopoietic cells and overexpression of HOXB4 results in amplified competitive repopulating ability in cotransplantation models in mice [25] and in extensive expansion of human hematopoietic stem cells ex vivo [6]. NF-Y is an essential factor for induction of HOXB4 expression in hematopoietic cells [26]. HOXB4 may be one of the targets of which the expression is disturbed in BM cells transduced with YAm29, leading to loss of expansion of the progenitor cells.

Apoptosis was specifically induced upon expression of YAm29 in the immature, MAC-1 negative, BM cells. This phenotype is consistent with the NF-YA conditional knock out studies done in primary MEF cells [16]. MEF cells that are devoid of functional NF-Y are blocked in their proliferation and undergo apoptosis.

NF-Y is a complex of three proteins. Overexpression of only the NF-YA subunit promotes the expansion of hematopoietic stem cells [17]. Here we show that inhibiting the function of the trimeric NF-Y complex, with a dominant negative protein, reduces the expansion of progenitor BM cells. Together, these data suggest that the increased expansion of immature BM cells upon overexpression of only the NF-YA subunit is through increased activity of the trimeric NF-Y complex. Therefore, the size of the pool of immature

hematopoietic cells may be regulated through
(de-)activating NF-Y.

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Isolation of FRET-positive cells using single 408 nm laser flow cytometry

Background: Flow cytometry may be used to isolate large amounts of living, fluorescently labelled cells. Certain fluorescent labels, like ECFP and EYFP, allow the assessment of direct protein-protein interaction, in situ, by FRET. However, current flow cytometric methods either require elaborate technical adaptations or, using a single laser protocol, are hampered by background signal. We optimized a single 408 nm laser protocol to detect FRET between ECFP/EYFP-tagged proteins.
Methods: Cell lines, stably expressing ECFP, EYFP, ECFP and EYFP or an EYFP-ECFP fusion protein, were used to design the settings for the flow cytometer to detect FRET-positive cells using a single 408 nm laser. Using these settings, interactions between the subunits of the transcription factor NF-Y were studied.
Results: Flow cytometric analysis of the cells expressing an EYFP-ECFP fusion protein, yielded a discrete FRET-positive population. Using the same settings, in cells expressing NF-YB-CFP and NF-YC-YFP fusion proteins, FRET could also be detected. These cells were sorted and FRET was confirmed by confocal microscopy.
Conclusion: FRET positive cells, expressing ECFP- and EYFP-tagged proteins, can be detected using single 408 nm laser excitation, with low background signal. This allows high-throughput analysis and isolation of viable FRET positive and negative cells for subsequent biological experiments.

Introduction

Fluorescence Resonance Energy Transfer (FRET) occurs when one fluorochrome (the donor) excites another fluorochrome (the acceptor) through direct resonance energy transfer. Upon excitation, the donor transfers its energy to the acceptor and the acceptor emits photons at its own characteristic wavelength. This phenomenon may occur when the emission spectrum of the donor significantly overlaps with the excitation spectrum of the acceptor. FRET is dependent on the physical distance between the two fluorochromes. Each donor/acceptor couple has a characteristic distance for which the FRET efficiency is 50%, meaning that 50% of the excited fluorochromes transfer their energy by FRET. This distance is usually between 30 and 60Å, which is roughly the size of a protein. The FRET efficiency will usually be less than 1%, and therefore undetectable, if the distance between donor and acceptor is more than 100Å (1-6).
In most biological assays FRET is measured using fluorescence microscopy (7,8). However, flow cytometry may offer some advantages. Using flow cytometry, large amounts of living cells can be studied in seconds. Additionally, cells of interest may be sorted for further biological studies. The determination of FRET with flow cytometry has been used to measure the interaction of cell surfaces molecules (9). In these experiments different antibodies, one coupled to a donor and one to a acceptor fluorescent dye, were used. Development of variants of the Green Fluorescent Protein (GFP) has made it possible to study intracellular proteins as well. A FRET couple that may be used in biological assays consists of the GFP variants, Enhanced Cyan Fluorescent Protein (ECFP; as the donor) and Enhanced Yellow Fluorescent Protein (EYFP; as the acceptor) (1,3). These

proteins can be cloned as fusion partner to any protein of interest. Because 50% FRET efficiency for ECFP/EYFP occurs at 50 Å (4), the fusion partners must be in close proximity to each other for this to happen. Therefore, FRET, using ECFP/EYFP, indicates that two proteins directly interact. These measurements can be done in living cells with proteins at their appropriate localization.

Flow cytometry has been used to study FRET between EYFP and ECFP (10-12). In the first two studies, the flow cytometer was equipped with two lasers and a set up using a time delay module and interlaser compensation to detect FRET. This allows, for each individual cell, the measurement of the amount of EYFP and ECFP emission due to direct laser excitation, but also the amount of EYFP emission due to FRET (FRET-EYFP). However, this setup is not easily applicable to all flow cytometers. The third study used a 458 nm laser to detect FRET. However, as the authors indicate, the drawback of this set up is that at this wavelength both EYFP and ECFP are excited. Fluctuations in EYFP expression will easily interfere with FRET detection in this protocol. When FRET efficiencies are low, most signal with 458 nm excitation will be from direct laser excitation of EYFP and not from FRET. Theoretically, a laser of approximately 400 nm would be preferable, as at this wavelength ECFP will preferentially be excited. We equipped a flow cytometer with a 408 nm laser and designed and tested a single laser flow cytometer protocol.

Materials and Methods

DNA vectors

pECFP-C1 and pEYFP-C1 were from Clontech (Palo Alto, CA, USA). NF-YA, -B and -C were cloned from pSG5 vectors (a kind gift of Dr. R. Mantovani, University of Milan, Italy) into pEYFP-C1 and pECFP-C1. The pSG5-NF-YA, pSG5-NF-YB and pSG5-NF-YC vectors were used as template in PCR reactions using forward primers containing a *BglII* restriction site and reverse primers containing an *EcoR1* site for directional cloning in the pEYFP/ECFP-C1 vectors.

ECFP-EYFP fusion construct

The EYFP-ECFP fusion construct was a kind gift of Dr. F.J. van Kuppeveld (UMC, Nijmegen) (13). The fusion protein separates EYFP from ECFP with a 35 amino acid linker. FRET efficiency for this construct has been determined with fluorescence lifetime imaging (FLIM). The fluorescence lifetime of a donor molecule (the time the molecule spends in the excited state) is reduced by FRET. Fluorescence lifetime imaging may be used to calculate FRET efficiency (6,14). A lifetime of 1.95 ns was described for ECFP in the EYFP-ECFP fusion construct, compared to a lifetime for free ECFP of 2.5 ns. This demonstrated a FRET efficiency of around 22% for the EYFP-ECFP fusion construct (13).

Cell culture and transfection.

Hep3B cells and HEK293 cells were cultured in D-MEM medium (Gibco, Gaithersburg, MD) supplemented with 10% fetal calf serum (Gibco). Transfections were done with calcium phosphate precipitates according to standard procedures. After 18-24 hours, cells were washed and harvested by trypsinization (Gibco) and taken up in HBSS (Cambrex, Verviers, Belgium). Transfections were done in a 1:1 molar ratio. The apparent FRET efficiency will be negatively influenced when donor molecules are present in higher concentrations than the acceptor molecules. Therefore, the relative expression of donor and acceptor molecules were monitored by flow cytometry and spectral imaging. In addition, the absolute expression levels were adjusted using appropriate concentrations of plasmid in order to minimize “false positive FRET” signal caused by direct EYFP excitation by the 408 nm laser.

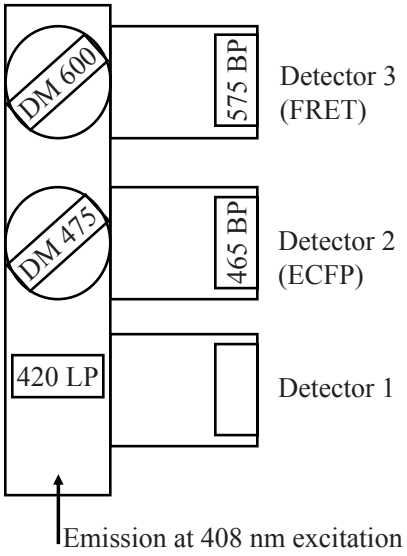

Figure 1. Flow cytometer settings for detecting FRET. Cells were excited using a 408 nm laser line. All outcoming signal under 420 nm was blocked using a 420 nm long pass filter in front of detector 1. Using a dichroic mirror, signal between 420 and 475 nm was directed to detector 2. Here, ECFP was detected using a 465±15 band pass filter. Using a dichroic mirror, signal between 475 and 600 nm was directed to detector 3. Here, EYFP was detected using a 575±15 nm band pass filter.

Flow cytometric analysis and sorting

Cells were analysed on a Epics Altra flow cytometer (Beckman Coulter, Miami, Florida). To asses the amount of EYFP expression, EYFP emission was detected at 575 nm after excitation with an 488 nm argon laser line. To detect FRET, the flow cytometer settings were as follows (Fig. 1): Excitation was with a diode, Vioflame, laser (408 ± 8 nm, Coherent Inc., Santa Clara, CA) running at 25mW. Signal below 420 nm was blocked with a 420 nm long pass filter. ECFP was separated from EYFP using a 475 nm dichroic mirror and ECFP was detected using a 465±15 nm band pass filter. FRET-EYFP was detected using a 600 nm dichroic mirror and a 575±15 nm band pass filter. Due to spectral overlap, ECFP will emit some light in the EYFP detector. Electronic compensation was set using cells transfected with ECFP only.

Acceptor Photobleaching FRET measurements

Acceptor photobleaching FRET microscopy was performed on a Zeiss LSM510Meta confocal microscope (Carl Zeiss GmbH, Jena, Germany) using a Plan-Apochromat 63x oil immersion objective with a numerical aperture (NA) of 1.4. Excitation of ECFP was accomplished using the 458 nm Argon laser line and ECFP emission was detected using a HFT458/514 main dichroic splitter, a NFT545 dichroic splitter, acting as short-pass filter and a 470-500 nm band pass filter. EYFP was excited using the 514 nm Argon line and the selective photobleaching of EYFP in acceptor photobleaching FRET experiments was accomplished by operating this line at full power. EYFP emission was detected using the same HFT458/514 and NFT545, now acting as long-pass filter, and a 535-590 nm band pass filter. Operating in line-wise multitrack mode, the ECFP and EYFP images were collected almost simultaneously and using this excitation/detection protocol the ECFP channel was essentially cross-talk free. Apparent energy transfer efficiencies E (6) were calculated from

$$E = 1 - \frac{I^{D}_{ROI,\,pre}}{I^{D}_{ROI,\,post}}$$

Here, $I^{D}_{ROI,\,pre}$ and $I^{D}_{ROI,\,post}$ refer to the intensity of the donor (D; ECFP) in a region of interest (ROI) before and after the selective photobleaching of the acceptor (EYFP).

The relative expression of donor and acceptor molecules in each cell was determined by spectral imaging analysis using the META detector (458 nm excitation, HFT458/514 main dichroic, spectral decomposition and

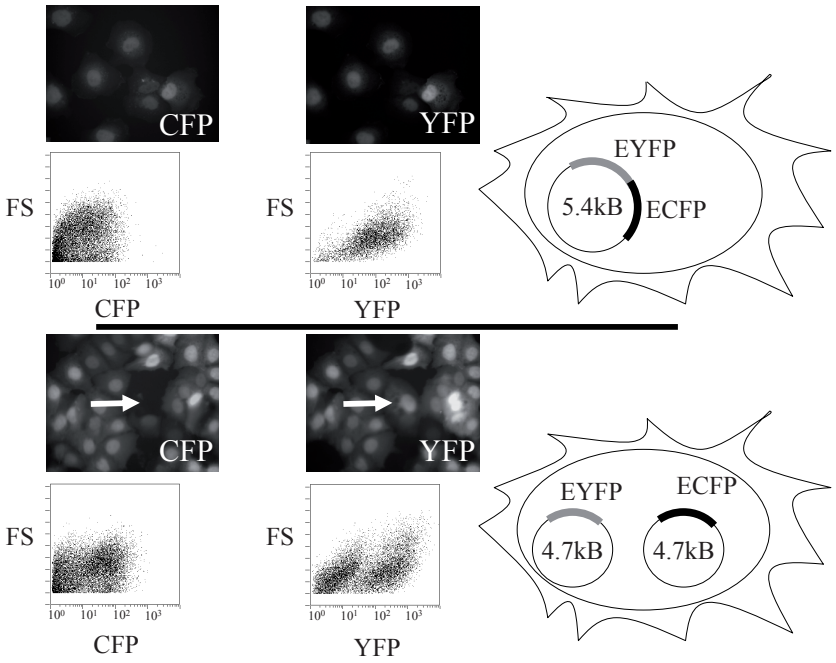

Figure 2. Detection of ECFP and EYFP expression in stably transfected Hep3B cells. Cells were transfected with an EYFP-ECFP fusion construct (upper panel) or two plasmids, one containing ECFP and one containing EYFP (lower panel). Cells were cultured and stable transfectants were selected by cell sorting. Cells were analysed by flow cytometry and fluorescence microscopy. Flow cytometric detection of ECFP was by excitation with a 408 nm laser line and EYFP was detected by excitation with a 488 nm laser line. ECFP and EYFP is plotted against forward scatter (FS).

subsequent detection using the 32 channel PMT array detector META). Despite the 1:1 molar transfection ratio, the spectral ratios deviate from 1 because FRET results in a lowered ECFP emission and enhanced EYFP emission. Typically, spectral ratios EYFP/ECFP (530/480) were 1.7 +/- 0.2 in the case of the ECFP-EYFP fusion protein, and 1.4 +/- 0.1 for the NF-YB-CFP and NF-YC-YFP co-expressing cells.

Results

To design the flow cytometer settings for detecting FRET-positive cells, four different cell cultures (Hep3B cells) stably expressing different EGFP variants were established by cell sorting, resulting in polyclonal cultures with cells expressing high to very low levels of the indicated proteins (Fig. 2). Two cultures were made that stably expressed either EYFP or ECFP alone. As a negative control for FRET, a culture was established expressing both EYFP and ECFP independently. As a positive control for FRET, a culture expressing an EYFP-ECFP fusion protein was made (see material and methods).

Characteristics of the cell cultures were determined using fluorescence microscopy. No difference was found in localization of

the fluorescent proteins between the cells expressing the EYFP-ECFP fusion protein and the ECFP and EYFP proteins separately (Fig. 2). Cells were analysed to check whether EYFP and ECFP were expressed at approximately equal amounts. Ratios and absolute levels were determined by a combination of spectral imaging and flow cytometry, as described in materials and methods. ECFP and EYFP transfected cells showed over 95% co-expression of both proteins in a comparable ratio. Because EYFP and ECFP do not bind to each other, this culture was used as a negative control for FRET studies.

The FRET efficiency of the cells expressing the EYFP-ECFP fusion construct was measured using confocal fluorescence microscopy and acceptor photobleaching. Because the donor molecule transfers part of its energy during FRET, it will emit more light in the absence of an acceptor molecule. Therefore, FRET can be detected by bleaching the acceptor molecule and measuring the increase in emission of the donor. EYFP was bleached in a selected region by operating a 514 nm laser line at full power. ECFP emission was measured before and after bleaching in the same region and FRET efficiency was determined by dividing these two values. This yielded a FRET efficiency for the EYFP-ECFP transfected cells of 21±5% (Fig. 3), which was comparable to the 22% efficiency measured by FLIM (see materials

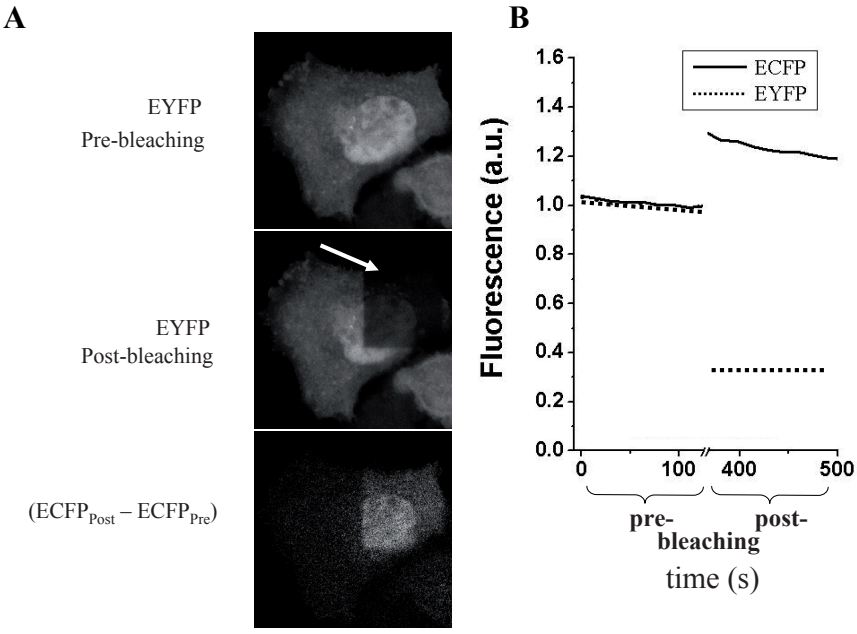

Figure 3. Detection of FRET by confocal microscopy in Hep3B cell expressing an EYFP-ECFP fusion construct. (A) Confocal microscopy was used to measure the intensity of ECFP and EYFP emission in cells expressing an EYFP-ECFP fusion construct. EYFP was bleached by selective photobleaching with a 514 nm laser line at full power. The arrow indicates the bleached region. The increase in ECFP emission after photobleaching was used to determine the FRET efficiency (see material and methods). In the lower image the ECFP emission intensity after bleaching is subtracted from the ECFP emission intensity before bleaching to visualise the increase in ECFP emission. The mean FRET efficiency was determined by bleaching several different cells. **(B)** The mean fluorescence intensity of the selected region, ECFP and EYFP, was monitored for 2 minutes before bleaching and 2 minutes after bleaching.

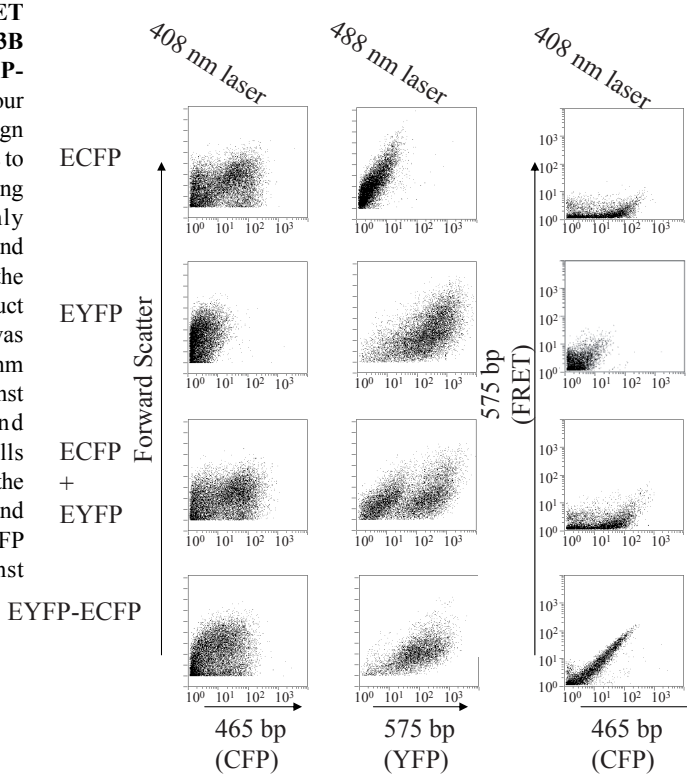

Figure 4. Detection of FRET by flow cytometry in Hep3B cell expressing an EYFP-ECFP fusion construct. Four cell lines were used to design the flow cytometry settings to detect FRET; cells expressing only ECFP (row 1), only EYFP (row 2), both ECFP and EYFP separately (row 3), the EYFP-ECFP fusion construct (row 4). EYFP emission was measured using the 488 nm excitation and plotted against forward scatter (second column). In the same cells FRET was measured using the 408 nm laser (column one and three). In column three ECFP emission is plotted against FRET-EYFP emission.

and methods). This indicated that these cells were a suitable positive control to design the flow cytometer settings to detect FRET.

To preferentially excite ECFP, we added a 408 nm laser to our flow cytometer. The ECFP expressing cells were used to determine the required electronic compensation, 465 detection out of 575 detection (not shown). Subsequently, this protocol was used for all acquisitions. Mean ECFP emission levels, and therefore expression levels, were comparable in the culture expressing ECFP alone or ECFP and EYFP separately (Fig. 4, first column). The EYFP-ECFP fusion protein expressing cells showed a slightly reduced level of ECFP emission in comparison to the other ECFP expressing cultures, compatible with the occurrence of FRET. At 488 nm excitation, EYFP emission levels were comparable in the cells expressing EYFP alone, EYFP and ECFP separately and cells expressing the fusion construct (Fig. 4, second column). At 408 nm excitation, EYFP emission could only be detected in the EYFP-ECFP fusion protein expressing cells (Fig. 4, third column), indicating FRET.

Next, we investigated whether we could detect FRET between two separate proteins that are known to interact. The transcription factor NF-Y consists of three subunits NF-YA, -B and -C. NF-Y is, as a complex, able to bind to CCAAT-boxes in the DNA and to the basal transcription machinery through binding of TBP (15,16). In this way NF-Y regulates the expression of many cell cycle related genes (17). NF-YA and NF-YB were fused to ECFP and all three subunits were fused to EYFP.

Different combinations of these constructs were transiently transfected and monitored for the presence of FRET using the same settings as described above. EYFP emission

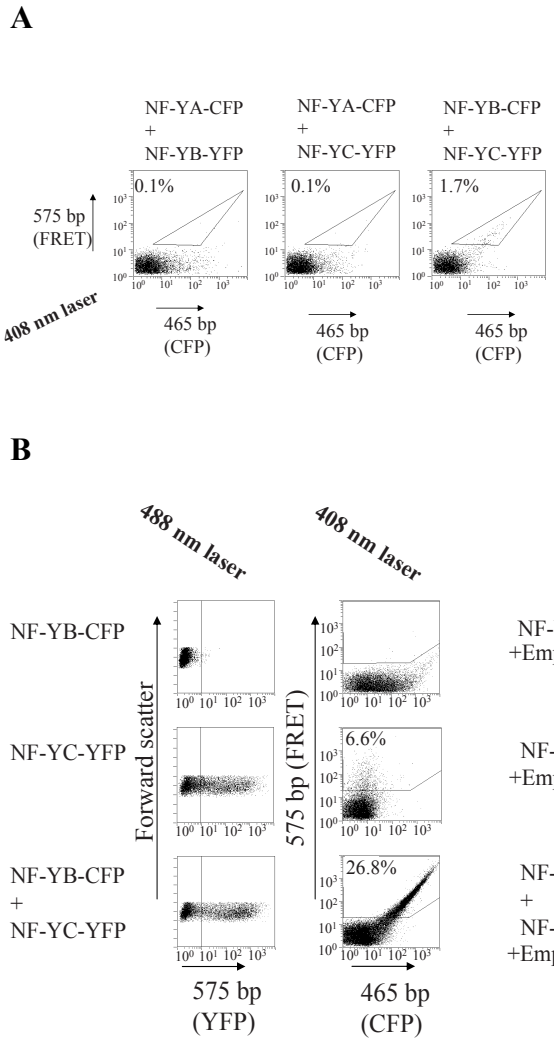

Figure 5. Detection and sorting of transiently co-transfected, FRET positive cells by flow cytometry. Cells were measured by flow cytometry using 488 nm excitation to detect EYFP. Using 408 nm excitation ECFP and FRET was detected. (**A**) Cells were transiently transfected with NF-YA-ECFP and NF-YB-EYFP or NF-YC-YFP or NF-YB-ECFP and NF-YC-YFP. (**B**) Cells were transiently transfected with NF-YB-CFP and/or NF-YC-YFP and untagged NF-YA (first two columns) or the same vectors were diluted 8 times with an empty vector (last two columns). EYFP was measured by excitation with the 488nm laser (column 1 and 3). ECFP and EYFP were measured by excitation with the 408nm laser (column 2 and 4).

was detected by excitation with the 408 nm laser in cells transiently transfected with NF-YB-ECFP and NF-YC-EYFP (Fig. 5A). This indicated that co-expression of NF-YB-ECFP and NF-YC-EYFP resulted in FRET. Excitation with the 408 nm laser did not result in EYFP emission in cells expressing NF-YA-ECFP and either NF-YB-EYFP or NF-YC-EYFP. Possibly, within the NF-Y complex, the ECFP-tag on NF-YA is too far apart from the EYFP-tag on NF-YB and NF-YC for FRET to occur.

The cell cultures described above, all expressed the EYFP proteins at comparable levels of between 1 to 30 times over background. We investigated whether FRET could still be detected in cells expressing extremely high levels of EYFP. Cells were transfected with NF-YB-CFP and/or NF-YC-YFP, with high efficiency and harvested after a two-day incubation. These cells showed high EYFP emission upon excitation with the 488 nm laser. (Fig. 5B, first row). Although FRET was detected in cells expressing NF-YB-CFP and NF-YC-YFP, some cells expressing only NF-YC-YFP were excited

with the 408 nm laser (Fig. 5B, second row). This indicated that high EYFP expression interfered with FRET detection. Diluting the transfection mixture with empty vector resulted in decreased expression levels of EYFP/ECFP-tagged proteins. In these cells a FRET signal was found only in cells expressing both NF-YB-CFP and NF-YC-YFP (Fig. 5B, compare column 1 and 2 with 3 and 4). This indicated that expression levels need to be under a threshold to get a pure FRET-positive population. This threshold was determined by making a dilution series of cells expressing high, to very low amounts of only EYFP (not shown). Analysis revealed that cells expressing less than 30 times over background, as measured with 488 nm excitation, were not detected in the EYFP/FRET channel upon 408 nm excitation. Taken into account this treshold, EYFP emission after dilution (figure 5B, last column) could only be explained by FRET.

To confirm that the cells transfected with NF-YB-CFP and NF-YC-YFP were FRET-positive, cells were sorted and cytospins were analysed by confocal fluorescence microscopy. Both cells expressing high levels of NF-YB-ECFP and NF-YC-EYFP and cells expressing low levels were sorted. FRET efficiency was 9 ± 1% in all cells, indicating that in all sorted cells, NF-YB and NF-YC interacted.

Discussion

The study of protein-protein interactions has been greatly improved by FRET microscopy, making it possible to study interactions *in situ,* within the living cell. Detecting FRET-positive cells by flow cytometry adds the possibility to analyse protein-protein interactions in living, non-homogenous cell cultures and allows the isolation of FRET positive and negative cells. Here, we describe a method to detect FRET-positive cells using single 408 nm laser flow cytometry. The protocol may be used to detect FRET in cells transfected with two plasmids encoding tagged-proteins of interest.

At 408 nm excitation, ECFP is near its absorbtion maximum, whereas EYFP is excited at a minimal level. Therefore, at this wavelength, EYFP excitation will interfere minimally with the detection of FRET. Using this protocol, FRET could be detected by monitoring the emission of EYFP. The flow cytometer settings were determined by making use of a construct in which EYFP is fused to ECFP. By two different methods the characteristics of this construct have been determined. FLIM demonstrated a FRET efficiency of around 22% for this construct (13). Photobleaching studies by confocal microscopy of cells, stably transfected with the ECFFP-EYFP fusion construct also confirmed that these cells were FRET positive, indicating that these cells were suitable to design the settings for a flow cytometry protocol.

Upon excitation with the 408 nm laser line a clear FRET-positive population could be detected in cells expressing a EYFP-ECFP fusion protein. Cells expressing only EYFP were not excited with the 408 nm laser line. This indicated that at the EYFP expression levels in these cells, direct excitation of EYFP with the 408 nm laser line did not interfere with FRET detection. Only cells expressing high amounts of EYFP gave some signal in the in the 575 nm detector (Fig. 5B). This indicated that with the 408 nm laser line EYFP was still somewhat excitated. Therefore, in FRET studies, using single 408 nm excitation it is necessary to use a control population of cells, expressing only the EYFP construct, that expresses equal amounts of EYFP as the FRET-positive cells. To accomplish this, transfection efficiency can, amongst others be controlled by diluting the transfection mixture with empty vector DNA.

The three subunits of NF-Y are able to form one complex. We were able to detect FRET between subunits NF-YB and NF-YC but not

between NF-YA and either NF-YB or NF-YC. This suggested that either tagged NF-YA was no longer able to form a complex with NF-YB and C, or that the orientation and distance between the tag on NF-YA and NF-YB or NF-YC were not optimal for FRET to occur. The detection of FRET with fluorescence microscopy in sorted cells expressing NF-YB-ECFP and NF-YC-EYFP confirmed that the 408 nm single laser can be used to detect FRET in cells expressing ECFP- and EYFP-tagged proteins from transiently transfected cDNAs.

In summary, we show that a 408 nm single laser is a ready-to-use tool to detect FRET, making it possible to isolate living FRET-positive and negative cells, expressing ECFP/EYFP-tagged proteins, for subsequent biological experiments.

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Summary and perspectives

Treatment of acute promyelocytic leukemia (APL) with high dose retinoic acid (ATRA) forces terminal differentiation of the leukemic blasts towards mature granulocytes with a normal, limited lifespan. This treatment constitutes one of the first clinically relevant differentiation-induction therapies in cancer. In this thesis we show that the PML-RARα fusion protein not only interferes with the transcription of regular retinoic acid receptor target genes, but may also interfere with the expression of Sp1-NF-Y regulated genes, defining a novel class of target genes and a gain-of-function for the oncoprotein.

PML-RARα is the causative oncogene in 5-10% of the cases of acute myeloid leukemia. The fusion protein interferes with the function of both non-rearranged PML and retinoic acid receptor-α counterparts. PML-RARα silences normal retinoic acid receptor target genes, but in response to high dose retinoic acid, it (re)activates the transcription of genes, forcing the cells into differentiation. We show that *ID1* and *ID2* are direct retinoic acid target genes in APL cells. The *ID1* and *ID2* promoters are activated by PML-RARα but, unexpectedly, not by wild type RARα/RXR. Our data support a model in which the PML-RARα fusion protein regulates a novel class of target genes by the interaction with Sp1-NF-Y transcription factors, without directly binding to the DNA.

We set out to identify genes that are responsive to ATRA and we investigated the role of these genes in ATRA-induced differentiation. In APL cells *ID1* and *ID2* are rapidly upregulated in response to ATRA, both at the mRNA and protein level. Treating cells with cycloheximide, an inhibitor of protein synthesis, did not inhibit induction of these genes, suggesting that PML-RARα directly regulated these genes. ID proteins are general inhibitors of basic Helix-Loop-Helix (bHLH) transcription factors. BHLH transcription factors are involved in the specification of the mesoderm during embryogenesis and are involved in the differentiation of mesodermal tissues like muscle, bone and blood (Nederbragt et al., 2002). The rapid upregulation of *ID1* and *ID2* in APL cells suggested a role for these proteins in APL cell differentiation. Therefore, we overexpressed ID1 and ID2 in the APL cell line NB4. Overexpression inhibited proliferation and induced a G0/G1 accumulation, indicating a role for ID1 and ID2 in ATRA-induced differentiation in APL.

Chapter 2. To investigate the mechanism by which PML-RARα regulates the transcriptional activation of *ID1* in response to ATRA, we cloned the *ID1* promoter in a luciferase reporter construct. Surprisingly, we found that the promoter was transactivated by PML-RARα in a retinoic acid dependent manner, but not by unrearranged RARα/RXR. To identify the regulatory DNA sequences through which the induction by PML-RARα was mediated, several deletion mutants were constructed. Deletion mutants that lacked all (even remotely) consensus retinoic acid response elements were still transactivated by PML-RARα. To test whether PML-RARα might transactivate the *ID1* promoter without directly binding to the DNA, we used a PML-RARα construct in which the DNA binding domain was deleted (PML-RAR/ΔR). While we confirmed that PML-RAR/ΔR was unable to transactivate a promoter containing a well-defined retinoic acid response element from the RARβ promoter, it could still transactivate the *ID1* promoter. This indicated that transactivation of *ID1* by PML-RARα occurred without direct binding of PML-

RARα to the DNA. When the coiled-coil domain of PML-RARα was deleted (PML-RAR/ΔCC) transactivation was abolished. As the coiled-coil domain is involved in protein-protein interactions, this suggested that the transactivation of *ID1* is dependent on homodimerisation and/or interaction with other protein(s) mediated through the PML-part of the chimera. Further deletion and point mutants identified the site in the *ID1* promoter that was necessary for PML-RARα mediated transactivation, showing that a CCAAT box (binding site for the transcription factor NF-Y) and adjacent GC box (binding sites for Sp1) were essential for transactivation.

NF-Y is a trimeric protein complex consisting of the subunits NF-YA, NF-YB and NF-YC. All three subunits are needed for the complex to bind to the DNA. When a dominant-negative form of NF-YA (YAm29) was used, transactivation of the *ID1* promoter by PML-RARα was abolished. To test whether PML-RARα could be recruited to the DNA through binding to either Sp1 or one of the NF-Y subunits, we performed GST-pull down experiments. These showed that PML-RARα may bind to Sp1, whereas no direct interaction with any of the NFY subunits was observed. Additionally, in chromatin immunoprecipitation assays, using anti-PML antibodies, we were able to precipitate the *ID1* promoter, both in U937 cells expressing PML-RARα and U937 cells not expressing PML-RARα, suggesting that both PML-RARα, and also the normal PML protein is present on the *ID1* promoter. From HEK293 cells, ectopically expressing FLAG-tagged PML-RARα, we were able to ChIP the *ID1* promoter, using anti-FLAG antibody. The data suggest a model in which PML-RARα binds to a DNA-bound SP1-NF-Y complex, rendering the expression of *ID1* sensitive to ATRA. The effect of PML-RARα on the endogenous *ID1* gene was tested using a U937 cell line that is stably transfected with a Zn^{2+}-inducible PML-RARα expression cassette. In PML-RARα expressing U937 cells, ATRA strongly induced *ID1* mRNA and protein expression, contrary to U937 cells lacking PML-RARα. Additionally, we found that the *ID2* gene, in response to ATRA, is regulated by the same mechanism as the *ID1* gene.

Together, this implicates that apart from deregulating RARα target genes, PML-RARα deregulates an additional class of genes. We show that the *HES1* gene is regulated in a comparable manner to *ID1* and *ID2,* suggesting indeed that the mechanism described is more general. The gain-of-function for PML-RARα may contribute to the successful treatment of acute promyelocytic leukemia.

Chapter 3. P53 has been implicated in the induction of apoptosis in genetically damaged cells, and p53-mediated eradication of cells is one of the major mechanisms protecting us against tumorigenesis. Disruption of p53 function is a common characteristic of cancer cells, and *TP53* is considered to be the most frequently mutated oncogene in human cancer. One of the most important negative regulators of p53 is the MDM2 protein. MDM2 binds p53, blocks p53-mediated transactivation and targets p53 for proteasomal degradation. In leukemia, p53 is rarely mutated, but its function may be deregulated by other mechanisms, for instance by overexpression of MDM2.

We tested whether PML-RARα expression would influence MDM2 expression. Induction of PML-RARα in U937 cells did not change MDM2 mRNA expression levels, but at the protein level, MDM2 was highly upregulated. Therefore, p53 may be deregulated in APL cells by increased levels of MDM2 protein. This mechanism may contribute to the transformation in APL.

A novel class of small molecules, Nutlins, was developed recently (Vassilev et al., 2004). Nutlins disrupt the binding of MDM2 to

p53, thereby inhibiting p53 degradation and activating p53-dependent apoptotic pathways. We analyzed the effect of Nutlins on primary APL patient samples. Cells from all APL patients showed, in variable degree, a dose- and time-dependent increase in apoptosis. We conclude that PML-RARα expression enhances MDM2 protein levels, and that APL cells are sensitive to MDM2 antagonists.

Chapter 4. As shown in chapter 2**,** PML-RARα may regulate gene transcription through binding of a Sp1-NF-Y DNA-bound complex. To determine the biological role of NF-Y in hematopoiesis we expressed a dominant negative form of NF-Y in murine bone marrow cells by retroviral transduction. Inhibition of NF-Y function resulted in a significant reduction of granulocytic (CFU-G) and monocytic (CFU-M) colony formation by 70% and 44 %, respectively. To study this reduction in more detail, we analysed the effect of dominant negative NF-Y on proliferation, differentiation and apoptosis. In more mature, MAC-1 positive cells, no differences in proliferation, differentiation and apoptosis were observed between dominant negative NF-Y- and vector- transduced cells. In contrast, the expansion of MAC-1 negative cells was completely abrogated by the expression of dominant negative NF-Y. Moreover, the inhibition of NF-Y function resulted in a significant increase in apoptosis in MAC-1 negative cells. Competitive transplantation of BM cells to irradiated mice, showed a repopulation advantage of vector-transduced cells versus dominant negative NF-Y transduced cells. This indicates that NF-Y regulates the expansion of the myeloid progenitor cells.

Chapter 5. To study the interaction of the different NF-Y subunits in living cells, we developed a flow cytometric protocol, to detect fluorescence resonance energy transfer (FRET). FRET occurs when one fluorochrome (the donor) excites another fluorochrome (the acceptor) through direct resonance energy transfer. FRET only occurs when molecules are close together and may be used to investigate whether proteins interact. We developed a flow cytometric protocol to detect FRET between ECFP/EYFP-tagged proteins. With this protocol we were able to detect FRET between NF-YB-CFP and NF-YC-YFP and we were able to sort the FRET-positive and FRET-negative cells. This protocol allows high-throughput analysis and isolation of viable FRET positive and negative cells for subsequent biological experiments.

PML-RARα in transformation. The transforming effect of PML-RARα may be explained by 4 mechanisms.

1) PML-RARα has a dominant negative effect on RARα. PML-RARα binds to RARα binding elements, resulting in silencing of RARα target genes. Wild-type RARα has a ligand-dependent and a ligand–independent activation domain. PML-RARα is able to repress the transcription of RARα target genes.

2) PML-RARα has a dominant effect on PML. PML-RARα binds and translocates the PML protein to an aberrant nuclear localization, antagonizing the role of PML in growth suppression and apoptosis. Additionally, PML-RARα may sequester PML binding partners and inhibit their function.

3) PML-RARα sequesters RXR. Apart from being a heterodimerization partner for RARα, RXR is a heterodimerisation partner for many nuclear hormone receptors. PML-RARα may block the function of these other nuclear hormone receptors by sequestering RXR.

4) The PML-RARα fusion protein regulates other genes besides RARα targets. PML-RARα homodimers bind to a larger variety of direct repeats than RARα/RXR. Additionally, PML-RARα binds to the DNA via a Sp1-NF-Y complex. PML-RARα may repress the transcription of these genes.

PML-RARα, its role in the ATRA-response. PML-RARα positive APL cells are sensitive to ATRA. In response to ATRA the APL cells differentiate into mature granulocytes and finally go into apoptosis. Recent studies show that APL cells are also sensitive to arsenic trioxide (ATO) (Douer and Tallman, 2005). ATO induces partial differentiation and, at higher dose, apoptosis of APL cells (Miller, Jr. et al., 2002). Common to both treatments is the induction of degradation of PML-RARα, suggesting that degradation of the oncoprotein is important for the response of the APL cells. In chapter 3 we show that APL cells are sensitive to Nutlins. We found that Nutlins induce a relative decrease in numbers of microspeckled cells and increase in numbers of cells with normal PML-nuclear bodies. FISH showed no relative decrease in numbers of cells with a t(15;17) translocation (data not shown). This suggests that also Nutlins induce degradation of the PML-RARα protein.

It is tempting to speculate that the APL cells differentiate and become apoptotic as a result of targeted degradation of the oncoprotein. However, several arguments indicate that PML-RARα is an active inducer of differentiation upon treatment with ATRA as well.

PLZF-RARα positive APL cells are, in contrast to PML-RARα positive APL cells, less sensitive to ATRA and only differentiate in the presence of other costimuli (Jansen et al., 1999). Still PLZF-RARα is, like PML-RARα, degraded in response to ATRA (Koken et al., 1999), suggesting that degradation of the oncoprotein is not sufficient for terminal differentiation in response to ATRA.

ATRA is a ligand for RARα. ATRA serves also as a ligand for PML-RARα and PLZF-RARα. However. PML-RARα only activates transcription in the presence of high-dose ATRA and PLZF-RARα does not activate transcription in response to ATRA because of an ATRA-independent co-repressor complex bound to the PLZF part of the fusion protein. This suggests that ATRA may activate genes in PML-RARα positive APL cells that are not activated in PLZF-RARα positive APL cells. These genes may be responsible for the induction of differentiation.

APL is the only type of AML sensitive to ATRA, suggesting an active role for PML-RARα in the ATRA response. An active role for PML-RARα in induction of differentiation in APL cells also follows from experiments showing that PML-RARα increases the sensitivity to ATRA when expressed in certain non-APL cells and restores ATRA-sensitivity in cell lines carrying mutations in RARα (Grignani et al., 1993; Grignani et al., 1996; Ruthardt et al., 1997). PML-RARα re-expression restores ATRA-sensitivity in APL cells with constitutive degradation of the fusion protein (Fanelli et al., 1999). Additionally, point mutations have been identified in the ATRA-binding domain of PML-RARα in ATRA-resistant APL patients.

Together these data suggest that activation of genes, in response to ATRA, by PML-RAR, results in the induction of differentiation in APL. The results from chapter 3 show that degradation of PML-RARα results in downregulation of MDM2 and subsequent apoptosis. Therefore, the terminal differentiation in response to ATRA is explained by the combination of sequential effects. First, activation of PML-RARα target genes results in differentiation of the cells and later, degradation of PML-RARα cancels the repressive effect on signalling pathways, resulting in apoptosis.

PML-RARα is able to target the same genes as normal RARα. The *p21*$^{(waf1/cip1)}$ promoter contains a retinoic acid response element, 1200 bp upstream of its transcription start site, and is a target of RARα/RXR (Liu et al., 1996). P21 is a cyclin-dependent kinase

inhibitor and uprgegulation of p21 causes cells to arrest in G_1 and withdraw from the cell cycle, suggesting that p21 is directly involved in ATRA-dependent differentiation. The RARβ has also been identified as target gene for RARα/RXR (de et al., 1990). However, no role for RARβ in APL differentiation has been described. Both genes are also transactivated, in response to ATRA, by PML-RARα.

In this thesis, we present a model showing a gain-of-function for the PML-RARα fusion protein. In response to ATRA, PML-RARα activates a class of genes that is not activated by normal RARα. This activation is dependent on binding of PML-RARα to the transcription factor Sp1. PLZF-RARα was, like RARα, unable to activate transcription of these genes, which underlines the importance of the PML-part of PML-RARα in the activation of transcription, and possibly in the induction of differentiation of APL cells. Interestingly, the normal PLZF protein may be able to interfere with binding of Sp1 to the DNA (Lee et al., 2002), suggesting PLZF-RARα may actively repress transcription of this class of genes.

We describe two target genes which are upregulated by PML-RARα in response to ATRA, by this gain-of-function. One of these target genes is *ID1*. We show that overexpression of ID1 in a hematopoietic cell line resulted in accumulation of these cells in the G0/G1 phase of the cell cycle. *C/EBP*β may be upregulated by the same mechanism as *ID1* is. Suppression of C/EBPβ expression in NB4 cells, using RNAi, results in a poor response of these cells to ATRA (Duprez et al., 2003). Therefore, both genes may be involved in the differentiating effect of ATRA.

An interesting experiment would be overexpression of C/EBPβ / ID1 / ID2 in AML cells of another FAB-type, and see whether these cells will be forced to differentiate. Alternatively, an interesting experiment would be expression of PML-RARα, *in vitro*, in primary AML cells of another FAB-type, and test whether these cells will be forced to differentiate and to test which genes become responsive to ATRA.

Relapse/ATRA resistant APL patients. Recent studies show that combined treatment of APL patients with ATRA and chemotherapy may result in long term disease free survival in 80-90% of the cases (Mistry et al., 2003). Although this percentage is very high, there is still room for improvement. Additionally, high dose chemotherapy may result in long term effects. Replacing chemotherapy with more selective or less genotoxic agents may be beneficial for APL patients. The following agents may be beneficial in the treatment of APL:

1) Durable remission is obtained following arsenic trioxide (ATO) treatment as a single agent. ATO induces apoptosis via multiple pathways and results in degradation of PML-RARα. The efficacy of ATO is currently under investigation.

2) PML-RARα is able to form stable complexes with HDACs, which may contribute to transformation by silencing of PML-RARα target genes (Grignani et al., 1998; Lin et al., 1998). Therefore, antagonizing HDACs with HDAC inhibitors, like valproic acid (VPA) and trichostatin A (TSA), could have potential in the treatment of APL. Recent studies have revealed that these inhibitors indeed posses antitumor activity (Insinga et al., 2005). Surprisingly, the mechanism behind this antitumor activity seems not dependent on PML-RARα expression but rather on the fully transformed phenotype of APL cells. Mice expressing PML-RARα develop leukemia after a long latency. During this latency, blasts of these mice are insensitive to HDAC inhibitors. However, mice with fully developed APL are sensitive to HDAC inhibitors. HDAC inhibitors were found to induce apoptosis, in APL blasts, by activation of the death receptor pathway, through upregulation of TRAIL, DR5, FasL and Fas.

3) There is evidence for malfunction in the p53 pathway in most cancers (Vogelstein et al., 2000; Vousden and Prives, 2005). In over 50% of the cases this is the result of a mutation in the *TP53* gene itself. However, in leukemia mutations in *TP53* are not very frequent (Hu et al., 1992). In this thesis we show that the MDM2 protein, one ot the negative regulators of p53, is upregulated by the PML-RARα oncoprotein. Specific inhibition of the MDM2-p53 interaction with the small molecule inhibitor Nutlin-3, resulted in apoptosis of APL blasts. These data indicate that Nutlin-3 may have therapeutical potential in APL.
4) Yondelis (ET-743) is a potent anti-cancer drug isolated from the marine animal *Ecteinascidia turbinata* currently under Phase lll clinical trials (D'Incalci and Jimeno, 2003). The anti-proliferative activity is more potent than taxol or etoposide. Many anti-cancer drugs influence gene expression by acting on specific promoters. Targets of Yondelis include NF-Y target genes. As the response to ATRA also includes NF-Y target genes, it would be interesting to study the effect of Yondelis on APL cells, or APL cell lines *in vitro*.

Open ends

PML-RARα in gene repression. PML-RARα, like RARα, functions as a ligand-activated zinc-finger transcription factor and is able to bind to specific sequences in the DNA. In the absence of ligand, PML-RARα is able to repress gene transcription by the recruitment of co-repressors (Alland et al., 1997; Heinzel et al., 1997; Nagy et al., 1997), resulting in methylation of DNA and deacetylation of histones, making the chromatin inaccessible to the transcriptional machinery. Active repression of target genes may be an important mechanism in transformation.
It remains to be investigated whether PML-RARα, complexed with Sp1-NF-Y, actively represses gene transcription by recruitment of co-repressors to the gene promoters of *ID1*. We studied the expression levels of ID1 in U937 cells, before and after introduction of PML-RARα. PML-RARα expression did not downregulate ID1 mRNA expression levels. However, mRNA expression levels of *ID1* were low, in comparison to expression levels after induction with ATRA (data not shown). At the protein level ID1 was not detectable in U937 cells, suggesting that ID1 may not be a good target to study repression.
C/EBPβ has three protein isoforms. The smallest isoform of C/EBPβ is detectable in U937 cells. This isoform is downregulated in response to expression of PML-RARα (data not shown). This suggests that PML-RARα may downregulate C/EBPβ mRNA in the absence of ligand, suggesting active repression of C/EBPβ. C/EBPβ plays an important role in granulocytic differentiation. Active repression of C/EBPβ may contribute to transformation in APL.

PML. One of the interesting outcomes of our studies is that the PML protein is present on the promoter of the *ID1* gene. PML expression has been shown to stimulate transcription of several genes. Additionally, PML interacts with chromatin remodelling factors, translation initiating factor 4E (Borden, 2002) and with the transcription factor Sp1 (Vallian et al., 1998). This suggests that PML may be a scaffold factor on the promoter of several genes, binding specific transcription factors, recruiting chromatin remodelling factors and loading the newly produced RNA with the first translation initiating factor. Future studies will have to determine the precise target genes of PML. These data may give a better insight into the role of PML in cell growth and apoptosis.

NF-Y. Recent studies have revealed that repression of several genes, by recruitment of HDAC activity to the promoter, is dependent

on NF-Y binding sites, suggesting NF-Y is directly involved in repression of several genes (Imbriano et al., 2005).
NF-Y may repress genes involved in the switch between self-renewal and maturation. Subsequently, expression of NF-Y may favour self-renewal, as seen upon overexpression of NF-Y in BM cells (Zhu et al., 2005) and inhibiting NF-Y may favour maturation. Moreover, activation of these targets by PML-RARα in APL cells upon treatment with ATRA may have the same effect as relieving the repression by NF-Y through expressing dominant negative NF-Y; induction of maturation and apoptosis, or in other words; terminal differentiation.

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Nederlandse samenvatting

Hoofdstuk 1 (inleiding). Dagelijks worden in het menselijk lichaam grote aantallen bloedcellen aangemaakt. Deze cellen beschermen het lichaam tegen infecties (immuniteit), vervoeren zuurstof door het lichaam en vormen stolsels bij verwonding. Bloedcellen ontstaan vanuit onrijpe cellen in het beenmerg. Deze onrijpe cellen worden, omdat ze kunnen uitrijpen tot alle verschillende soorten bloedcellen, stamcellen genoemd. Dit proces heet hematopoïese. In leukemie is de uitrijping van cellen geblokkeerd. Deze blokkering leidt tot ophoping van onrijpe cellen in het beenmerg en het verdringen van de normale hematopoïese.

In acute promyelocytenleukemie (APL) is de cel geblokkeerd in het stadium van de promyelocyt, de cel die normaal gesproken uitrijpt tot granulocyt, een celtype betrokken bij afweer. APL wordt "acuut" genoemd vanwege de snelle progressie van de ziekte welke zonder behandeling snel fataal afloopt.

In 1988 publiceerden Huang *et al.* een studie waaruit bleek dat APL patienten gevoelig zijn voor een afgeleide stof van vitamine A (retinol): all-*trans* retinoic acid. Deze stof dwingt de APL cellen tot terminale uitrijping, zowel *in vitro* als ook in patiënten. Combinatie van all-*trans* retinoic acid met chemotherapie leidt bij meer dan 80% van de APL patienten tot een overleving langer dan 5 jaar. Dit maakt APL, tot op heden, de best behandelbare vorm van acute leukemie.

In bijna alle gevallen van APL wordt dezelfde genetische afwijking gevonden: een uitwisseling tussen de lange armen van chromosoom 15 en 17. Deze uitwisseling resulteert in een fusie tussen de genen *PML*, op chromosoom 15 en het *retinoic acid receptor* α gen (*RAR*α), op chromosoom 17. Expressie van dit fusie-gen in gezonde muizen veroorzaakt APL, waarmee wordt aangetoond dat *PML-RAR*α het causale oncogen is bij deze ziekte.

*RAR*α codeert voor een ligand-afhankelijke transcriptiefactor. RARα bindt specifieke sequenties in het DNA en activeert specifieke target genen in aanwezigheid van ligand: retinoic acid. In APL is de functie van RARα verstoord. Het PML-RARα fusie-eiwit is nog steeds in staat om specifieke sequenties in het DNA te binden. Echter, om genen te activeren is voor PML-RARα een meer dan 100 maal hogere concentratie retinoic acid nodig dan voor RARα. Dit suggereert dat genen die in een gezonde situatie geactiveerd worden door fysiologische concentraties retinoic acid, bij APL niet geactiveerd worden. Behandeling van APL patiënten met een hoge dosis retinoic acid resulteert wel in activatie van deze genen en uitrijping van de leukemische cellen.

Welke genen worden geactiveerd door retinoic acid in APL? Antwoord op deze vraag zou kunnen helpen het uitrijpingsproces in APL te begrijpen en zo mogelijk moleculaire targets geven voor onderzoek naar behandeling van andere vormen van leukemie.

De *ID1* en *ID2* genen coderen voor remmers van zogenaamde basic Helix-Loop-Helix (bHLH) transcriptiefactoren. Een rol voor ID-eiwitten en bHLH-eiwitten is aangetoond in de uitrijping van verschillende soorten weefsels. We onderzochten het effect van ID-eiwitten op de uitrijping van APL. Behandeling van APL-cellen met retinoic acid resulteerde in een directe inductie van ID1 en ID2. Deze uitkomst suggereerde een rol voor ID1 en ID2 tijdens APL uitrijping. Daarom brachten we ID1 en ID2 tot overexpressie in een APL-cellijn. Deze overexpressie resulteerde in een remming van de proliferatie en een ophoping van de cellen in de G0/G1 fase van de celcyclus. De data suggereren dat ID1 en ID2 een rol spelen bij het therapeutische effect

van retinoic acid op APL cellen.

Hoofdstuk 2. Om de rol van PML-RARα in de inductie van *ID1* te onderzoeken, kloneerden we de promoter van *ID1* in een luciferase reporter construct. Tot onze verbazing vonden we dat PML-RARα de *ID1* promoter kon transactiveren, in tegenstelling tot het normale RARα dat de *ID1* promoter niet kon transactiveren. Dit is in tegenspraak met de theorie, die zegt dat PML-RARα en RARα dezelfde target genen hebben. Om de DNA-sequenties te vinden die betrokken zijn bij de transactivatie, maakten we verschillende deletiemutanten van de *ID1* promoter. Deletiemutanten, die alle stukken misten die ook maar zijdelings op de consensus RARα-binding sequentie leken, werden nog steeds getransactiveerd door PML-RARα en niet door RARα. Om uit te zoeken of PML-RARα de *ID1* promoter kon transactiveren zonder direct DNA te binden gebruikten we een PML-RARα construct waar het DNA-binding domein gedeleteerd was. Deze PML-RARα mutant was nog steeds in staat de *ID1* promoter te transactiveren. Dit gaf aan dat transactivatie mogelijk gebeurde zonder direct DNA contact. Deletie van het coiled-coil domein van PML-RARα resulteerde in het volledig teniet doen van de transactivatie. Aangezien het coiled-coil domein betrokken is bij eiwit-eiwit interacties, suggereert dit dat de transactivatie afhankelijk is van homo-dimerisatie en/of interactie met andere eiwitten. Verdere deletie en mutatieanalyse van de *ID1* promoter toonden aan dat een CCAAT-box (bindingsplek voor de transcriptiefactor NF-Y) en aangrenzende GC-box (bindingsplek voor de transcriptiefactor Sp1) essentieel waren voor de transactivatie. Het verhinderen van de functie van NF-Y, met behulp van een dominant negatieve mutant van NF-Y, verhinderde de transactivatie van de *ID1* promoter door PML-RARα. Met behulp van GST-pull down experimenten werd een interactie tussen PML-RARα en Sp1 gevonden, maar geen interactie tussen PML-RARα en NF-Y. In chromatine immunoprecipitatie assays werd met behulp van anti-PML-antilichamen de *ID1* promoter geprecipiteerd. Gezamelijk wijzen deze resultaten op een model waarin PML-RARα een Sp1-NF-Y complex bindt op de *ID1* promoter, waardoor de *ID1* promoter gevoelig wordt voor retinoic acid.
Het effect van PML-RARα op de endogene *ID1* promoter werd getest in de U937 cellijn die stabiel getransfecteerd is met een Zn^{2+} induceerbare expressie cassette. In aanwezigheid van PML-RARα werd, na behandeling met retinoic acid, een versterkte respons van *ID1* gevonden.
We tonen tevens aan dat het *ID2* gen en het *HES1* gen, via hetzelfde PML-RARα afhankelijke mechanisme als *ID1* gereguleerd worden. De data impliceren dat PML-RARα, naast het dereguleren van RARα target genen, een additionele klasse van genen dereguleert. Deze 'gain-of-function' draagt mogelijk bij aan het succesvolle effect van retinoic acid in de behandeling van APL.

Hoofdstuk 3. P53 speelt een rol in de inductie van celcyclus arrest en apoptose, celdood, in genetisch beschadigde cellen. Het p53-gemedieerde opruimen van cellen is één van de belangrijkste mechanismen die ons beschermen tegen kanker. Verstoring van de p53 functie is een veel voorkomend fenomeen in kanker cellen en *TP53* is, voor zover nu bekend, het meest frequent gemuteerde gen in kanker.
Een negatieve regulator van p53 is MDM2. MDM2 bindt p53, verhindert p53 genen te transactiveren en markeert p53 voor proteasomale afbraak. In leukemie is *TP53* zelden gemuteerd, echter, zijn functie is mogelijk gedereguleerd door andere mechanismen, bijvoorbeeld door overexpressie van MDM2.

We hebben onderzocht of PML-RARα door verstoring van MDM2 expressie, de p53 functie remt. Inductie van PML-RARα in de U937 cellijn resulteerde niet in verandering van het mRNA expressie niveau van MDM2. Inductie van PML-RARα gaf echter wel een verhoogd niveau van MDM2 eiwit. Dit betekent dat p53 mogelijk gedereguleerd wordt in APL cellen door een verhoogd eiwit niveau van MDM2.
Een nieuwe klasse van 'kleine moleculen', Nutlins, is onlangs ontwikkeld. Nutlins verstoren de binding van MDM2 met p53, en verhinderen daarmee de afbraak van p53 en maken p53-afhankelijke apoptose mogelijk. We hebben het effect van Nutlins op primaire APL cellen getest. Cellen van APL patiënten vertoonden een dosis- en tijdsafhankelijke toename in apoptose in response tot Nutlins. We concluderen dat PML-RARα expressie het MDM2 eiwit niveau verhoogt en dat APL cellen gevoelig zijn voor Nutlins.

Hoofdstuk 4. Zoals we in hoofdstuk 2 aantonen dereguleert PML-RARα target genen van NF-Y. We hebben daarom de functie van NF-Y onderzocht in de hematopoïese. Met behulp van retrovirale transductie werd een dominant negatieve mutant van NF-Y tot expressie gebracht in beenmerg stamcellen van muizen. Expressie van deze mutant verhinderde de normale functie van NF-Y en remde, *in vitro*, de koloniegroei van granulocyten (CFU-G) en monocyten (CFU-M) met respectievelijk 70% en 44%.
Om deze remming in detail te bestuderen, analyseerden we het effect van dominant negatief NF-Y op proliferatie (groei), differentiatie (uitrijping) en apoptose (celdood). In meer rijpe, MAC-1 positieve, cellen was geen verschil te zien in proliferatie, differentiatie en apoptose tussen dominant negatief NF-Y getransduceerde cellen en controle cellen. De expansie van onrijpe, MAC-1 negatieve, cellen was volledig geremd in dominant negatief NF-Y getransduceerde cellen en niet in de controle cellen. Ook nam apoptose toe in deze onrijpe fractie na expressie van dominant negatief NF-Y. Competitieve transplantatie van beenmergcellen in bestraalde muizen leverde, ten opzichte van dominant negatief NF-Y getransduceerde cellen, een groei-voordeel op voor de controle cellen. De data tonen aan dat functioneel NF-Y nodig is voor de expansie van onrijpe myeloide cellen.

Hoofdstuk 5. Fluorescence Resonance Energy Transfer (FRET) is het fenomeen dat een fluochroom (de donor) een ander fluochroom (de acceptor) kan exciteren. Voorwaarden voor deze energie overdracht is dat het emissiespectrum van de donor overlapt met het excitatiespectrum van de acceptor en dat de donor en acceptor minder dan 10 nanometer van elkaar verwijderd zijn. Met behulp van FRET-analyse kan bestudeerd worden of 2 eiwitten een interactie aangaan, door één van deze eiwitten te fuseren met een donor- en de ander met een acceptorfluochroom. In dit hoofstuk staat een techniek beschreven die FRET-analyse koppelt aan flow cytometry. Deze techniek maakt het mogelijk om, binnen seconden, duizenden cellen te analyseren op FRET en de cellen op FRET-positiviteit en –negativiteit te sorteren, voor mogelijk verdere biologische experimenten.
NF-Y bestaat uit een complex van 3 eiwitten: NF-YA, -B en -C. Deze eiwitten moeten aan elkaar binden om een een actief NF-Y complex te vormen. We tonen aan dat het mogelijk is FRET te meten in cellen die getransfecteerd zijn met NF-YB en NF-YC, gekoppeld aan respectievelijk een donor en acceptor fluochroom. Met het boven genoemde protocol was het mogelijk de FRET-positieve en FRET-negatieve cellen te sorteren.

Acute promyelocytenleukemie en verder onderzoek. Ondanks het relatief hoge genezingspercentage voor APL, is er nog steeds ruimte voor verbetering. Patiënten die niet goed reageren op therapie zouden mogelijk met andere stoffen geholpen zijn. Bovendien worden momenteel nog hoge dosis chemotherapie gegeven naast de retinoic acid behandeling. Deze behandeling kan op lange termijn nog schadelijke gevolgen hebben. Vervanging van chemotherapie door een selectievere stof, of een minder toxische stof, zou verbetering kunnen geven in de behandeling van APL patiënten. Voorbeelden hiervan zijn:

1) *Arsenicum trioxide*. APL cellen zijn gevoelig voor arsenicum trioxide. Deze stof induceert uitrijping van APL cellen en, in hogere dosis, apoptose. Behandeling van APL cellen resulteert onder andere in afbraak van het PML-RARα tumor-eiwit. Momenteel wordt de effectiviteit van arsenicum trioxide bij de behandeling van APL in verschillende studies onderzocht.

2) *HDAC remmers*. PML-RARα voorkomt de activatie van target genen onder meer door het rekruteren van HDAC eiwitten. Dit mechanisme is betrokken bij de transformatie van APL. Het remmen van HDAC eiwitten zou daarom potentie kunnen hebben in de behandeling van APL. Recente studies hebben aangetoond dat een aantal HDAC remmers inderdaad antitumor activiteit hebben. Het mechanisme achter deze antitumor activiteit lijkt echter niet afhankelijk van PML-RARα expressie, als wel van het volledig getransformeerde APL fenotype. HDAC remmers induceren apoptose, in APL cellen, door middel van het activeren van de 'death receptor pathway'.

3) *Nutlins*. Er is bewijs voor het niet functioneren van p53 in de meeste tumoren. In meer dan 50% van de gevallen komt dit door een mutatie in het *TP53* gen zelf. In leukemie komen deze mutaties echter veel minder frequent voor. In dit proefschrift laten we zien dat de concentratie MDM2, de negatieve regulator van p53, verhoogd wordt door het PML-RARα tumoreiwit. Specifieke remming van de p53-MDM2 interactie, met behulp van Nutlins, resulteert in apoptose van APL cellen. De data tonen aan dat Nutlins potentie hebben voor de behandeling van APL.

4) *Yondelis* (ET-743) is een veel belovende antitumor geneesmiddel. Deze stof wordt geïsoleerd uit het zeedier *Ecteinascidia turbinata* en wordt momenteel onderzocht in Fase III klinische studies voor soft-tissue carcinoma's. De antiproliferatieve activiteit van Yondelis is groter dan die van taxol of etoposide. Yondelis beïnvloedt de expressie van specifieke genen, onder andere NF-Y target genen. Aangezien de NF-Y target genen ook betrokken zijn bij de response van APL cellen op retinoic acid, zou het interessant zijn het effect van Yondelis op APL cellen te testen.

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1995-2001:	Biology at the University of Utrecht. First internship entitled; "*A lophotrochozoan twist gene is expressed in the ectomesoderm of the gastropod mollusk Patella vulgata"* at the Department of Developmental Biology, University of Utrecht. Second internship entitled; "*Interactions of eukaryotic initiation factors with the cytoskeleton*" at the Biochemistry Department at the University of Sussex, England. Final Essay entitled; "*Bacteriophagetherapy*".
2001-2006:	PhD student at the Central Hematology Laboratory at the University Medical Centre Nijmegen. Thesis entitled; "*Downstream effects of the PML-RARα fusion in acute promyelocytic leukemia*".
2006-now:	Postdoc at the genomics laboratory at the University Medical Centre Utrecht. Study focuses on signal transduction and transcription using the model system *Saccharomyces cerevisiae.*

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1.De transcriptiefactor NF-Y is betrokken bij het reguleren van de aantallen onrijpe cellen in het lichaam. (dit proefschrift)

2.Het eiwit MDM2 is een mogelijk target voor therapie bij acute promyelocytenleukemie. (dit proefschrift)

3.Het fusie-eiwit PML-RARα heeft meer directe target-genen dan de nucleaire receptor RARα. Deze genen zijn betrokken bij differentiatie van bloedcellen, en dus mogelijk ook bij het therapeutische effect van ATRA in acute promyelocyten leukemie. (dit proefschrift)

4.Algemeen voorkomende mutaties in kanker zijn apoptose en signaal-transductie/transcriptie gerelateerd. Apoptose kan alleen in complexe meercellige organismen onderzocht worden. Echter, voor een beter begrip van signaal-transductie/transcriptie is de ééncellige gist, dankzij eenvoudige handelbaarheid en manipuleerbaarheid, het systeem bij uitstek.

5.Niet toevallig is de kloosterling Mendel de oervader van de genetica: genetica was en blijft monnikenwerk.

6.Hoe weinig van wat aanvankelijk in de verbeelding bestond wordt ooit werkelijkheid.

7.Het publiceren van een papieren versie van een proefschrift is in ons digitale tijdperk een dure en achterhaalde traditie.

8.In de weerbarstige werkelijkheid markeert een proefschrift niet zelden het einde van een wetenschappelijke carrière.

9. Zowel de blauwdruk van de mens als ook de afdruk van de mens is een chromosoom (zie afbeelding Y.Klein).

10. Het dichten van Gerrit Achterberg, in zijn zoektocht naar de geliefde dode, is experimenteren in vitro, hetgeen zich in vivo maar niet realiseren laat.

11. Niet het vinden, het zoeken is de beloning.

Yves Klein
Anthropometrie